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BONES, MUSCLES AND VITAMIN C

III. REPAIR OF THE EFFECTS OF TOTAL DEPRIVATION OF VITAMIN C AT THE PROXIMAL ENDS OF THE TIBIA AND FIBULA IN GUINEA-PIGS

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The changes to be described occurred at the proximal ends of tibial and fibular diaphyses of guinea-pigs which were first subjected to a period of total deprivation of vitamin C, and then supplied with the vitamin. The methods and material used are described in the first paper of this series (Murray & Kodicek, 1949*a*, p. 158), and the experiments are summarized in Table 1 of that paper (p. 160).

The changes occurred in two phases: a destructive phase followed by one of repair.

THE DESTRUCTIVE PHASE

This phase occurred only in animals which suffered a period of total deprivation of vitamin C.

It is well known that the pathological changes in scurvy include the cessation of ossification at the growth cartilage and elsewhere, the appearance of microfractures at the ends of the diaphyses of long bones, and even the detachment of epiphyses. Since it was our object to keep the animals alive until well after the time when the vitamin was put back into the diet, we have little histological material which would enable us to confirm the classical descriptions of these effects of total deficiency; we have, however, radiographs. Those, made immediately or shortly after the end of the period of total deficiency, show unmistakable evidence of the changes mentioned. In normal guinea-pigs, radiographs show the proximal end of the tibial diaphysis widening gradually to the growth cartilage, like a trumpet to its mouth, and the epiphysis fitting neatly over the diaphysis without overlapping it (Text-fig. 1A, and first paper, Pl. 1). On the other hand, in many guinea-pigs of groups 3, 4, 5 and 6 of Exps. 5 and 6, the first radiographs, made after 9 days of total deprivation of vitamin C followed by seven (Exp. 6) or eight (Exp. 5) days on 0·5 mg. ascorbic acid daily, showed the proximal epiphysis of the tibia overlapping the diaphysis medially, or both medially and laterally, while the diaphysis itself more or less completely lacked its normal proximal expansion (Text-fig. 1B, Pl. 1, figs. 1A, 2A, 3A, Pl. 2, fig. 12A). The change was usually greater on the medial than on the lateral side. It was observed in nineteen of the fifty-four legs. Similar changes were seen in Exps. 7 and 8.

* This work was done while the first author was at the Department of Biology, St Bartholomew's Hospital Medical College.

In addition, the epiphysis, which in normal animals sits squarely on the top of the diaphysis in a plane at right angles to the long axis of the shaft, was in many cases tilted downward, nearly always on the medial side (Pl. 1, fig. 1, etc.). Since it retains its normal relation, through its articular surfaces, ligaments, etc., with the femur, its sloping attachment to its own diaphysis causes the animal to be bow-legged; compare Pl. 1, fig. 4, a pair of normal limbs, with fig. 5. Sloping epiphyses were seen in nineteen of the fifty-four legs, and nearly always in the same legs which also showed epiphyseal overlap.

The loss of the diaphyseal expansion, and the medial slope of the epiphysis, were accompanied by the appearance of an irregular shadow usually extending across the diaphysis immediately below the growth cartilage, and by the presence, in the angles between the end of the diaphysis and the overhanging edge of the epiphysis, of small spicule-like shadows strongly suggestive of outwardly fractured bits of the proximal end of the diaphysis.

From this picture, and from the classical descriptions of scurvy in guinea-pigs, it was clear that the gently widening proximal end of the tibial diaphysis had been destroyed, that it was now represented by debris which gave the X-ray shadows just mentioned, and that the region of the diaphyseal wall now in contact with the epiphysis was not its normal proximal end (Text-fig. 1A), but a narrower region which would normally be situated more distally. Hence the epiphyseal overlap (Text-fig. 1B). The slope of the epiphysis obviously reflects more extensive destruction of the medial wall of the tibia than of the lateral wall; the lateral wall is probably to some extent protected by the fibula whose proximal epiphysis is in guinea-pigs fused with that of the tibia.

Radiographs taken later in the experiment may show the condition aggravated, sometimes in an extreme degree; thus, the diaphysis might fracture almost right across just below the growth cartilage (Pl. 3, fig. 14B). Such severe changes were rare, but there was often an increase in the medial downward slope of the epiphysis. By far the commonest and most interesting changes, however, were constructive, and led to the formation of the structures described below.

THE PHASE OF REPAIR

The reparative changes occurred only in those animals to which, after the period of total deprivation, a supply of vitamin C was again allowed. The amount of the vitamin given varied in the different experiments (Exps. 5–8, Table 1); it is of interest that the repair occurred when the amount of ascorbic acid provided was 0·5 mg. daily, apparently as well as when it was 10 mg., although the smaller amount did not usually allow normal formation and consolidation of fracture calluses; subperiosteal thickenings, too, remained unconsolidated.

In radiographs taken at various times after the restoration of the vitamin to the diet, the proximal end of the tibial diaphysis showed thickenings of its wall which sometimes appeared to be purely periosteal in origin (Pl. 1, figs. 1, 2) but sometimes also endosteal. The new bone thus formed tended to fill in the gap between the overhanging epiphysis and the diaphysis below it, and often appeared to form a callus-like mass within the diaphyseal cylinder just below the growth cartilage. The general effect was to restore the tibia to something like its normal form; but the epiphyseal

slope and resulting bow-leggedness were not corrected during the course of our experiments.

We studied these changes almost exclusively in the tibia, giving but little attention to the fibula, in which changes of a generally similar character appear to have occurred, because it is much easier to study and interpret the alterations in the larger bone. The radiographs gave no indication that similar changes, either of a destructive or of a reparative character, occurred at the distal ends of these bones, and we cut no sections of this region.

Sections were prepared of fifteen representative specimens. They were mostly frontal serial sections through the proximal ends of the tibia and fibula, but some included the distal end of the femur and some were cut in other planes. The material was all taken from animals which, after a period of total deficiency of vitamin C, were again supplied with the vitamin. Table 1, in conjunction with Table 1 of the first paper (p. 160) (Murray & Kodicek, 1949*a*) will indicate sufficiently the history of these animals.

Table 1.

Exp.	Group	No. of animals	No. of days from day 1 to death	No. of days from restoration of vitamin C to death
5	3	3	63, 113, 121	45, 95, 103
	4	2	45, 62	27, 44
	6	1	61	43
6	4	2	30, 85	14, 69
7	1	2	62, 64	52, 54
	2	3	107 (3 animals)	97 (3 animals)
8	2	2	66 (2 animals)	50 (2 animals)

It will be simplest to begin the description of the structural changes revealed in the sections by describing specimen 'VA 40' which died earliest after restoration of the vitamin. This animal died 13 days after transference from a diet without vitamin C to one containing 0·5 mg. ascorbic acid daily. It is of interest because it still shows, in some degree, the histological counterpart of the destructive changes which the radiographs indicate as occurring during the total deprivation of the vitamin, not yet obscured by the effects of the added ascorbic acid, and because it also shows earlier stages in the processes of repair than are found in animals which survived longer. The sections (Pl. 1, figs. 7, 8; Pl. 2, fig. 9) are frontal and show the tibial wall, both laterally and medially, not continuing as it normally does (Pl. 1, fig. 6; Text-fig. 1 A) to the level of the growth cartilage, but ending below it in a fibrous connective tissue ('Fasergerüst') which locally replaces the normal haematopoietic marrow. In this tissue, scattered near the upper end of the tibial shaft, are small pieces of bone. Some of these seem to be degenerating; we believe them to be detached fragments of the diaphyseal wall, broken off by microfractures during the total lack of the vitamin, or soon afterwards. Pl. 1, fig. 8 and Pl. 2, fig. 9 show similar fragments evidently just broken off, one in the tibia and one in the fibula. Also lying in the same connective tissue are islands of development of new bone of the granular type mentioned in the second paper (Murray & Kodicek, 1949*b*) in connexion with the mid-diaphyseal thickenings and their development.

Occupying the extreme proximal end of the tibial marrow cavity are lightly built bony trabeculae, many of them enclosing vestiges of the matrix of the growth

cartilage, at which they have evidently been formed by endochondral ossification. This bone must have been formed since ossification started again after restoration of ascorbic acid to the diet. The endochondral trabeculae here formed are being strengthened by the deposition of endosteal bone on their surfaces and by the formation of new endosteal trabeculae.

The tibial shaft showed (Pl. 1, fig. 7), both laterally and medially, a widened periosteal cambium with new bone forming in it just as in the development of a mid-diaphyseal thickening. This new bone was not revealed by the radiographs and so was presumably incompletely calcified.

The widened cambial tissue ended proximally at a mass of cartilage (Pl. 1, fig. 7, *peri.cart.*) which, like the new subperiosteal bone, is not normally present. This cartilage, which is seen both laterally and medially, lay between the fibrous layer of the periosteum and the bony shaft and callus, at the proximal end of the tibial diaphysis. Its tissue passed by a gradual transition into that of the periosteum from which it was evidently formed. The internal cells of the cartilage were hypertrophied and the matrix was basophil, while those nearer the surface were small and lay in an almost colourless matrix (haematoxylin and eosin). Radiographs showed that the matrix was calcified. In places the cartilage had a tenuous connexion with the epiphyseal cartilage which thus had the appearance of being turned downwards at its rim, around the end of the diaphysis, much as a match-head grips the stick. It is not being resorbed and no endochondral ossification is occurring in it.

The condition of the fibula, in the region of the growth cartilage, in general resembles that of the tibia; in particular, it shows a very beautiful microfracture of the bony shaft (Pl. 2, fig. 9).

We thus see in this specimen: (*a*) a widened subperiosteal cambium with new bone developing in it; (*b*) a bony callus-like tissue forming endosteally in the splintered proximal end of the tibial shaft; (*c*) the formation of trabecular bone from the growth cartilage; and (*d*) a differentiation of cartilage from the periosteum in the same region.

When we turn to the remaining specimens of which sections were cut (Table 1), all of which survived for longer periods than that just described, we find that their radiographs showed a great variety in the amount of reparative hard tissue formed at the proximal ends of the tibiae. Thus, in the specimen illustrated in Pl. 2, fig. 10B the medial wall of the tibia showed a thickening which increased towards the proximal end, stopping rather abruptly a little below the growth cartilage, but showed no other change except the medial slope of the epiphysis. It is notable that the thickening ceases proximally at a position corresponding with that of the top of the diaphyseal wall in the first photograph (fig. 10A) (taken just before the end of the total deficiency), as indicated by the distance from the point at which the medial wall of the diaphysis curved laterally. Pl. 1, fig. 1A, B, show somewhat greater changes, with subperiosteal new bone running up to the epiphysis on both sides, a medial slope of the epiphysis, and a suggestion (confirmed by sections) of endosteally formed callus. The bones shown in Pl. 2, fig. 12A, B; Pl. 3, fig. 13A, B, and fig. 14A, B show generally similar but greater changes: thickening of the proximal tibial wall in relation to epiphysial slope, overlap caused by the destruction of the proximal ends of the tibial diaphyses, and a suggestion of internal callus formation. In the specimen shown in Pl. 3, fig. 14A, B the extreme degree of over-

lap of the epiphysis on the medial side, caused by breakdown of the diaphysis, led to a fracture which sections show to have been nearly completely across the bone.

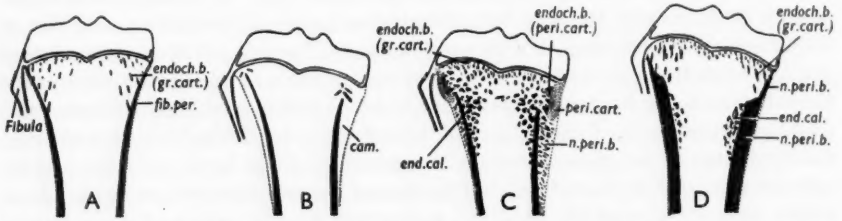
Sections show that the changes in these and other specimens must be referred to the four processes discerned at work in the specimen which died earliest, and which is described above. We shall discuss these processes *seriatim*.

Formation of the subperiosteal thickening

In the specimen which died at 13 days ('VA 40') after transference to a diet containing vitamin C, the subperiosteal thickening developed in a widened cambium. In other specimens, all of which lived longer after restoration of the vitamin, comparison of the earlier radiographs with the structure revealed by the sections, showed that a periosteal thickening had formed in the same way. In these sections it was possible to distinguish the bone formed in the subperiosteal thickening, and that of the endosteal callus developed in the marrow cavity, from the old bone of the original diaphyseal shaft, by the more regular orientation of the cells and fibrous structure of the old bone and by its staining properties, in which it differed slightly from the more recently formed bone. In spite of resorptive changes the old diaphyseal wall could usually be traced far enough towards the growth cartilage for it to be obvious by extrapolation that it pointed, not to the edges of the growth cartilage, but to places within the perimeter of the latter (Pl. 2, fig. 12D, E). The sections thus confirmed what the earlier radiographs showed, that at an earlier time the epiphysis had overlapped the proximal ends of the diaphysis. Now, the periosteum normally ends proximally where its fibres pass into the epiphyseal cartilage, just proximal to the growth cartilage, and into the joint capsule; from this attachment it normally descends along the diaphysis, lying close against it. But evidently when the diaphysis has lost its proximal end, the periosteum must tend to be loose over the angle between the diaphysis and the overlapping epiphysis. Now, after a fracture of the shaft of a normal guinea-pig's fibula the damaged periosteum re-establishes itself around a cellular mass which develops in the oedematous and haemorrhagic area between and around the broken ends. Because of the local haemorrhage and oedema, the newly re-established periosteum is displaced outwards from the bone on which, therefore, the callus forms a thickening. Similarly, the oedema developing in connexion with the damaged proximal end of the tibial diaphysis must tend to swell the periosteum locally outwards. Thus the anatomical changes brought about in the destructive phase, and the resulting oedema, combined to produce a widened subperiosteal space around the proximal end of the diaphysis; this space filled with osteogenic cambial tissue, and the proximal thickening resulted. It was clear from the relation of the periosteum to the angle beneath the overhanging epiphysis and to the diaphysis that the new bone thus formed tended to re-establish the normal widening of the proximal end of the tibial shaft (see Text-fig. 1B).

It would be supposed that the new bone would have functional value in supporting the overlapping epiphysis and (in terms of theories of functional determination of bone structure) might be regarded as brought into being by mechanical stimulation of a functional nature. While it is obvious that the new bone might have the functional value suggested, in that its trabeculae could make direct contact with the epiphysis and transmit strain from it, three considerations forbid us attributing its formation

to this stimulation. First, sometimes such contact seemed to be lacking, any strains carried from the epiphysis to the new bone being transmitted to it through connective tissue which seemed quite unfitted to such a function. Secondly, the trabeculae tended to be orientated radially and downward, from the old bone to the fibrous layer, and this is a direction roughly perpendicular to that expected if they were weight-carrying piers. Thirdly, in the early stages of its development the new bone could hardly discharge such a function because it was little if at all calcified; a bony structure can only develop in response to mechanico-functional stimulation if either the strains develop *pari passu* with the bone, or if, as in endochondral ossification, the bone takes over the function of some pre-existing structure which has hitherto borne the strain.



Text-fig. 1. Diagram of the changes at the proximal end of the tibial diaphysis in an animal supplied with vitamin C after a period of total deprivation. A. Proximal end of normal tibia and fibula; compare Pl. 1, fig. 6. B. After a period of total deprivation of vitamin C; microfractures at the proximal end of the tibial diaphysis reduce its length, especially laterally; the epiphysis is made to slope downward laterally, and the cambial zone (*cam.*) of the periosteum is widened. C. During repair. New subperiosteal bone (*n.peri.b.*) forms in the widened cambium. Periosteal cartilage (*peri.cart.*) and endosteal callus (*end.cal.*) are seen. Endochondral bone (*endoch.b.*) is forming by replacement of the periosteal cartilage and from the growth cartilage. Compare Pl. 1, fig. 7. D. The new subperiosteal bone has been made compact, periosteal cartilage has been resorbed, and the endosteal callus partly so; normal activity of growth cartilage. The most recently formed part of the diaphyseal wall (at the top) is of the normal thickness. Compare Pl. 4, fig. 16C. Fibrous layer of periosteum (*fib.per.*) dotted.

The development of subperiosteal thickenings on the fibula might be somewhat differently determined; Pl. 2, fig. 9 shows the development of new bone in an angle between periosteum, epiphysis, and old bone, created by a bend in the fibula at the level of the growth cartilage.

The periosteal cartilage

The cartilage was always formed at the top of the diaphysis and below the growth cartilage, internal to the periosteum and external to the proximal end of the diaphysis or to the callus in which the diaphysis ends (Text-fig. 1C). It was formed by local chondrification of the periosteum, into which it passed peripherally by a gradual transition. It was usually continuous with the cartilage of the epiphysis, and often looked as if formed by the epiphyseal cartilage being bent round, or having flowed round, the end of the shortened diaphysis; this was suggested, for example, in Pl. 2, fig. 9. We do not think, however, that this appearance can indicate an actual histogenetic process because of the clear evidence of periosteal origin of the cartilage and because of the difficulty in supposing the differentiated epiphyseal cartilage to possess such great mobility.

The cartilage appeared in about half the tibiae studied, and especially in those showing the greatest disturbance of the normal structure. As in the specimen which died earliest ('VA 40'), the cells in the middle of the cartilage hypertrophied and the matrix here became basophil; in specimens kept for longer periods the hypertrophy spread to the surface facing the bone and here the cell chambers were opened and endochondral ossification occurred (Pl. 3, fig. 15B). Peripherally, where the cartilage passed over into the periosteum, the cells remained small and the matrix eosinophil, or at least the enlargement and basophilia did not occur till later. The bone formed by replacement of this cartilage was added to the endosteally formed callus in which the old diaphyseal bone could often be seen to end (Text-fig. 1C). Its extent could be estimated from the vestiges of cartilage matrix embedded in the trabeculae of bone, and in one or two cases the presence of these revealed that a part of the callus had developed in a cartilage which had otherwise been totally destroyed by the time of fixation.

The cartilage must be compared with that formed in most fracture calluses, also from the periosteum or its derivative cells. There is strong evidence that connective tissue structures which do not normally form cartilage can be caused to do so by rather ill-defined mechanical conditions in which they are subjected to pressure. This has been shown for embryonic material *in vitro* by Glücksmann (1939), and, in tendons subjected to lateral pressure, by Ploetz (1937–8), while Krompecher (1937) found that cartilage formation at experimental fractures was prevented when the broken ends were pulled apart. In the present experiments it has repeatedly been seen, in both dietetically normal and partially deficient animals, that where the tibia and fibula come into contact and presumably press on each other, the periosteum of both chondrifies. It is thus possible, and in accord with other evidence, that the chondrification of the periosteum at the proximal end of the tibial diaphysis may be a reaction to the pressure of the internal oedema upon it. It is perhaps significant that it formed at the extreme proximal end of the shaft, where microfractures and resorptive changes deprived the periosteum of protection by an intact diaphyseal wall beneath it from the action of pressures acting from within the bone. Possibly, it may have been a reaction to pressure downwards from the epiphysis, but it had not this appearance. The consequence of its formation and later replacement by bone was to add to the bony callus which, with the new subperiosteal bone, the endosteal callus, and the bone formed by renewed activity of the growth cartilage, completed the restoration of the connexion between the epiphysis and the diaphyseal shaft. Further, since the cartilage was formed from the periosteum, its replacement by bone continued the widening of the new proximal end of the shaft, and therefore helped to restore the normal form of the tibia.

Endosteal callus

This was trabecular bone developed endosteally, especially about the shattered upper end of the tibial diaphysis. It formed a large part of the callus developed in this region, the remainder being formed partly by replacement of the periosteal cartilage and partly by endochondral ossification at the growth cartilage. Often, especially when it is large, the callus develops in the fibrous connective tissue by which the haematopoietic marrow is partly replaced, but sometimes it forms in

unaltered haematopoietic tissue. The quantity of endosteal callus formed differed greatly in different specimens, presumably depending on the severity and detailed nature of the injury. It might extend right across the marrow beneath the growth cartilage, or for a considerable distance along the inner aspect of the shaft wall, limiting the extent of the marrow cavity (Pl. 2, fig. 12C, Pl. 3, figs. 13C, 15A; and compare Pl. 4, fig. 18 with fig. 17, normal). We can offer no explanation of its occasional extent so far from the seat of injury.

When the old bone of the diaphysis had been so far destroyed as not to constitute a barrier between them, the endosteal callus and the subperiosteal thickening united, but the approximate position of the boundary between them could usually be detected by the more regular radiating arrangement of the subperiosteal trabeculae, those of the endosteal bone being usually much less regularly orientated.

In animals which lived for long periods after the restoration of vitamin C to the diet, it was sometimes found that the callus lay at some distance below the proximal end of the diaphysis; in such cases the callus must have formed at the proximal end soon after the restoration of the vitamin and then, comparatively normal growth being resumed, a new length of shaft was formed above it by the growth cartilage and periosteum (Text-fig. 1D; Pl. 4, fig. 16C).

The activity of the growth cartilage

The growth cartilage, which must be supposed to have been inactive during the period of total deprivation of the vitamin, in the sections showed resumed activity and endochondral bone was seen in formation. This activity, because of the diameter of the cartilage, must evidently have led to the development of a new proximal end of the diaphyseal shaft, and this must evidently have had the diameter of the growth cartilage, i.e. a diameter greater than that of what had, during the destructive phase, become the proximal end of the shaft. The formation of a fairly smooth junction between the wide new proximal part of the shaft, and the narrower old part now just distal to it (Text-fig. 1B, C, D), was brought about partly by the new subperiosteal bone, partly by the new bone formed endochondrally at the periosteal cartilage, partly by the endosteal callus, and partly of course by the modelling process which normally brings about the transformation of the wider proximal parts of the shaft into the narrower more distal part.

At first, the bone formed at the growth cartilage contributed to the internal callus, but as growth proceeded the cartilage was carried away from the callus, and a gap appeared between the recently formed endochondral bone and the internal callus. This gap had presumably earlier contained endochondral bone which had been resorbed, as occurs in normal growth (internal spongy bone being present only at the ends of most long bones).

A similar effect could be observed in connexion with the subperiosteal thickening; thus, in Pl. 2, fig. 10C the most proximal part of the lateral wall of the shaft was of about normal thickness, but at a short distance from the growth cartilage the wall abruptly thickened. This thickening was caused by subperiosteal bone developed as usual beneath an epiphysis overhanging a shortened and therefore abnormally narrow diaphysis; but, as soon as the growth cartilage became active and formed

a diaphyseal end of normal diameter, no widened subperiosteal space existed and as the new wall was formed it was of normal thickness.

SUMMARY

1. When guinea-pigs were subjected to total deprivation of vitamin C they showed the classic changes and, since the proximal end of the tibial diaphysis was destroyed by repeated microfractures, the epiphyses came to overlap, both laterally and medially, the narrow zone of the tibial shaft now in contact with it. The damage to the diaphysis was usually greater medially than laterally, causing the epiphysis to slope downwards and medially, making the animals bow-legged.

2. When animals, in which these changes had occurred during a period of total deficiency, were again given the vitamin, reparative changes restored the tibia to a form approaching the normal. These changes were: (*a*) the formation of a subperiosteal thickening in the widened periosteal cambium which filled the angle between the overhanging epiphysis above, the fibrous layer of the periosteum, and the old diaphyseal wall; (*b*) the formation, around the proximal end of the diaphysis, of cartilage derived from the periosteum, and its later replacement by endochondral bone which was added to (*c*) a trabecular bony callus developed endosteally; and (*d*) trabecular bone formed endochondrally at the growth cartilage. All these changes occurred in some cases, but not in every case, and there was great variation in detail.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. From a partially vitamin C-deficient guinea-pig of Exp. 5, group 4. A. 27th day of experimental diets, 9th day on 0·5 mg. ascorbic acid daily, day of operation (fracture just visible in original). On the medial side, the proximal epiphysis overlaps the diaphysis, which lacks its normal curvature. B. 47th day of experimental diets, 20 days after operation, 29th day on 0·5 mg. ascorbic acid daily, day of death. Thickening of proximal end of tibial diaphysis, medial slope of epiphysis, no radiologically recognizable callus at fracture.

Fig. 2. From a partially vitamin C-deficient guinea-pig of Exp. 5, group 6. No operation. A. 27th day of experimental diets, 9th day on 0·5 mg. ascorbic acid daily. The proximal end of the tibial diaphysis has lost the normal outward flare of the walls, and a heavy shadow lies below the epiphysis. B. 44th day of experimental diets, 26th day on 0·5 mg. ascorbic acid daily. Subperiosteal thickening of both tibial and fibular diaphyses, except distally; medial slope of tibial epiphysis which overlaps diaphysis medially. C. 61st day on experimental diets, 43rd day on 0·5 mg. ascorbic acid daily, day of death. No epiphyseal overlap because of increased proximal thickening of tibial diaphysis.

Fig. 3. From a partially vitamin C-deficient guinea-pig of Exp. 5, group 4. A. 27th day on experimental diets, 9th day on 0·5 mg. ascorbic acid daily, day of operation. Tibial epiphysis overlaps diaphysis medially, and on this side the diaphyseal wall has lost its curvature. B. 45th day of experimental diets, 27th day on 0·5 mg. ascorbic acid daily, 19th day after operation. Epiphysis detached from tibia, diaphyseal wall of tibia thickened near proximal end, no callus at fracture.

Fig. 4. The knees and tibio-fibulae of a normal animal.

Fig. 5. The knees and tibio-fibulae of a partially vitamin C-deficient guinea-pig, from Exp. 7, group 1, 62nd day of experimental diets, 52nd day on 0·5 mg. ascorbic acid daily, showing the bent knees, making the animal bow-legged. Contrast fig. 4. Figs. 4 and 5 are at the same magnification, and the scale between them is in centimetres.

Fig. 6. From a normal guinea-pig; frontal section of the proximal end of the tibia and fibula. Azan. ×9.

Fig. 7. From a partially vitamin C-deficient guinea-pig ('VA40') of Exp. 6, group 4, 30th day of experimental diets, 14th day on 0·5 mg. ascorbic acid daily, 6th day after operation. Frontal section of proximal end of tibio-fibula, showing an early stage in the development of new subperiosteal bone, periosteal cartilage, and endochondral and endosteal callus developing in the connective tissue which replaces the normal haematopoietic bone marrow. Haematoxylin and eosin. ×13.

Fig. 8. From the same partially vitamin C-deficient guinea-pig as fig. 7, showing a microfracture at the top of the tibial diaphysis; above and to the right, periosteal cartilage. Haematoxylin and eosin. ×210.

PLATE 2

Fig. 9. From the same partially vitamin C-deficient animal as fig. 7. Frontal section of the proximal end of the fibular diaphysis, showing a microfracture and new subperiosteal bone developing in a widened cambium. Haematoxylin and eosin. ×62.

Fig. 10. From a guinea-pig of Exp. 8, group 2, allowed cabbage after 16 days total deprivation of vitamin C. A. 15th day of total deficiency. Tibial epiphysis overlaps diaphysis medially. B. 64th day of experiment, 48th day on cabbage. The tibial epiphysis shows a slight medial slope, and the diaphysis a thickening of its medial wall beginning a little below the growth cartilage. C. 66th day of experiment, 50th day on cabbage. Frontal section of proximal end of tibio-fibula; the tibial wall is thickened medially except just below the growth cartilage. Haematoxylin and eosin. ×7.

Fig. 11. From a partially vitamin C-deficient guinea-pig of Exp. 5, group 4. 47th day of experimental diets, 29th day on 0·5 mg. ascorbic acid daily, 20th day after operation. See also radiographs of this animal in Pl. 1, fig. 1. Part of frontal section of tibia, showing callus at top of the diaphysis, periosteal cartilage being resorbed, and new subperiosteal bone (low right). Haematoxylin and eosin. ×17.

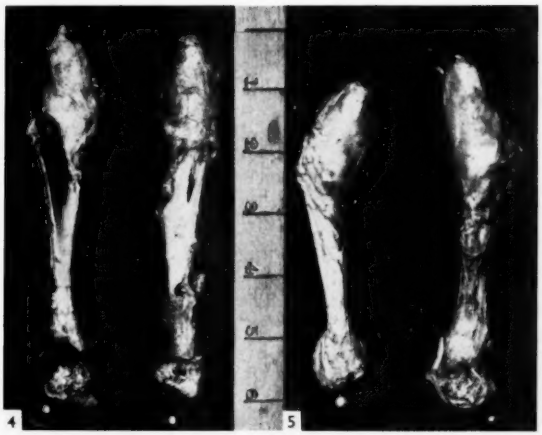
Fig. 12. From a partially vitamin C-deficient guinea-pig of Exp. 5, group 4. A. 27th day of experimental diets, 9th day on 0·5 mg. ascorbic acid, day of operation. Tibial epiphysis overlaps diaphysis on both sides; diaphysis lacks its normal proximal widening. B–E. 63rd day of experimental diets, 45th day on 0·5 mg. ascorbic acid daily, 37th day after operation, day of death. Great subperiosteal and endosteal thickening of diaphyseal wall; epiphysis no longer overlaps diaphysis. C. Frontal section of proximal end of tibia and fibula, showing immense thickening of diaphyseal walls and reduction of marrow cavity. Haematoxylin and eosin. ×7. D. Portion of medial wall of section near that shown in C, showing periosteal cartilage, the remains of the original diaphyseal wall outlined in black, and callus. Haematoxylin and eosin. ×17. E. Resembles D, but from the lateral wall; the dark area in the middle of the callus is unresorbed cartilage. Haematoxylin and eosin. ×17.

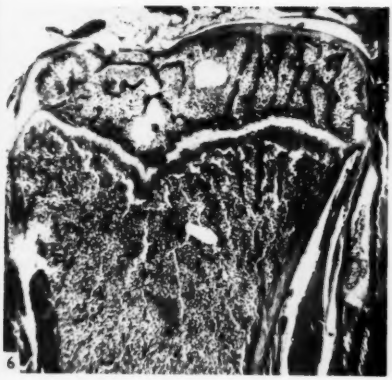
PLATE 3

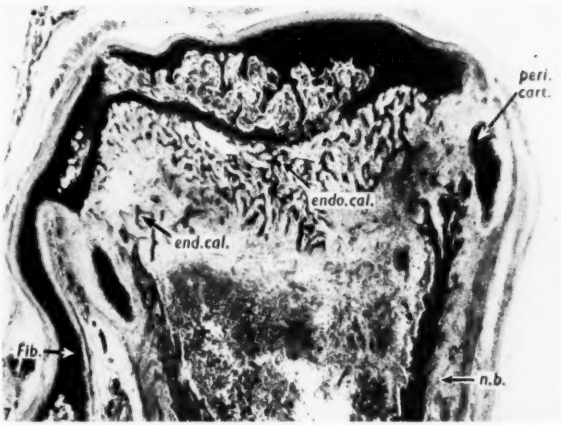
Fig. 13. From a partially vitamin C-deficient guinea-pig of Exp. 7, group 1. A. 15th day of experimental diets, 5th day on 0·5 mg. ascorbic acid daily. Tibial epiphysis overlaps diaphysis medially. B, C. 62nd day of experimental diets, 52nd day on 0·5 mg. ascorbic acid daily. Medial slope of epiphysis; thickening of both diaphyseal walls. C. Haematoxylin and eosin. ×7.

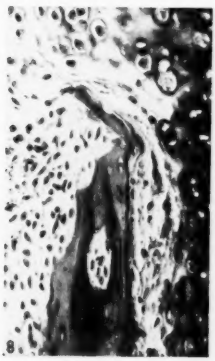
Fig. 14. The same animal as fig. 13, photographs taken on same days as fig. 13 A and B, but of the other leg. A. Great medial overlap of tibial epiphysis. B. Extreme epiphyseal slope due to collapse of proximal end of diaphysis.

Fig. 15. From a partially vitamin C-deficient guinea-pig of Exp. 7, group 1. 64th day of experimental diets, 54th day on 0·5 mg. ascorbic acid daily. A. Frontal section of proximal ends of tibia and fibula; the tibial diaphysis shows immense thickening of the wall, reduction of the marrow cavity, and periosteal cartilage. Haematoxylin and eosin. ×6. B. The area in the black square in fig. A more highly magnified, showing endochondral bone formation in the periosteal cartilage, and the

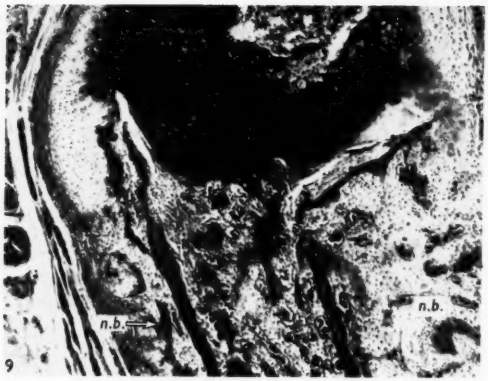








MURRAY AND KODICEK—BONES, MUSCLES AND VITAMIN C



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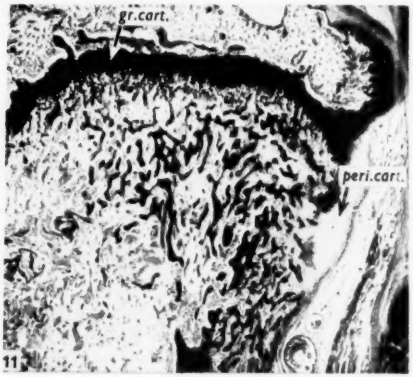

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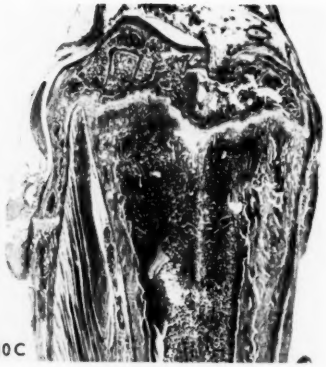
10A

10B



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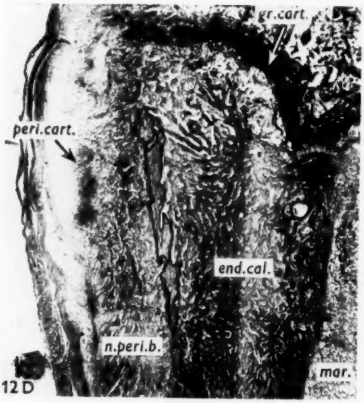
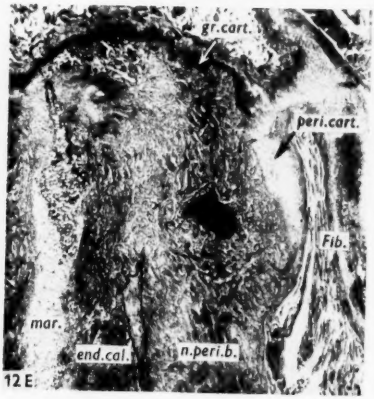


12D



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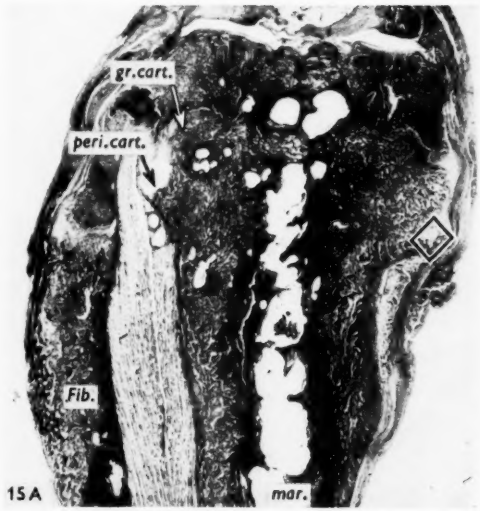
MURRAY AND KODICEK—BONES, MUSCLES AND VITAMIN C

13A
13B

14A
14B

13C


gr.cart.
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Fib.
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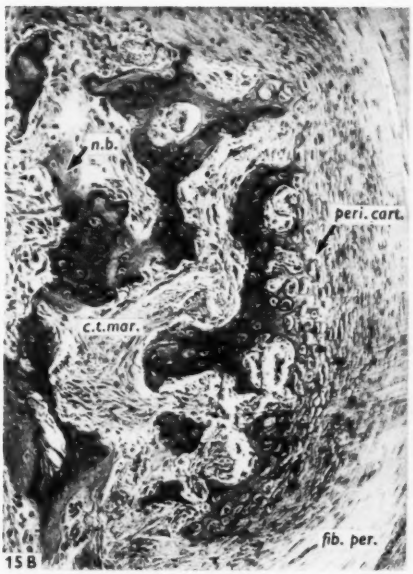
15A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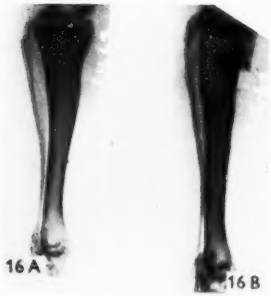
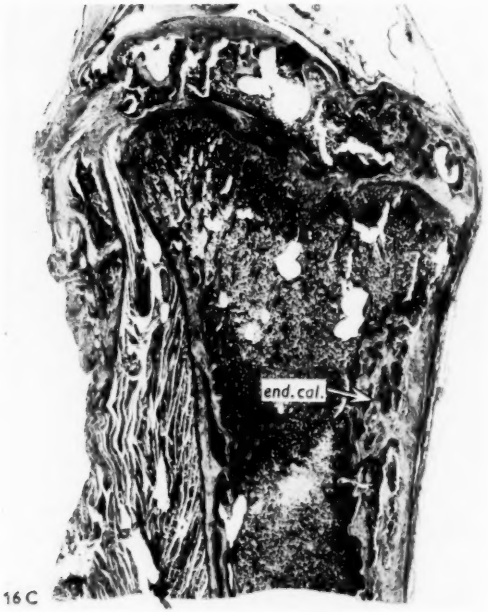
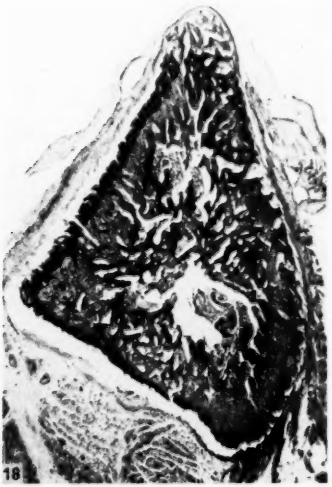

16 A
16 B


end. cal.
16 C

17


18

latter passing over into periosteum (from a section close in the series to A). Haematoxylin and eosin. × 150.

PLATE 4

Fig. 16. From a guinea-pig of Exp. 8, group 2, allowed cabbage after 16 days total deprivation of vitamin C. A. 15th day of total deprivation of vitamin C; slight medial slope of tibial epiphysis. B. 64th day of experiment and 48th day on cabbage. C. Frontal section of proximal end of tibia, 66th day of experiment, 50th day on cabbage, showing medial slope of epiphysis and endosteal callus on the medial side, a little below the growth cartilage. Haematoxylin and eosin. × 7.

Fig. 17. From a normal guinea-pig. An oblique section across the tibial diaphysis near its proximal end. Haematoxylin and eosin. × 12.

Fig. 18. From a partially vitamin C-deficient guinea-pig of Exp. 5, group 3. 121st day on experimental diets, 103rd day on 0·5 mg. ascorbic acid daily. The marrow cavity is almost obliterated by the endosteal callus. Compare fig. 17, a section which in position and orientation closely agrees with that here shown. Haematoxylin and eosin. × 11·5.

Abbreviations

c.t.mar.	connective tissue of marrow ('Fasergerüst').
end.cal.	endosteal callus.
endo.cal.	endochondral callus.
Fib.	fibula.
fib.per.	fibrous layer of periosteum.
gr.cart.	growth cartilage.
mar.	marrow.
n.peri.b.	new subperiosteal bone.
peri.cart.	periosteal cartilage.
n.b.	new bone

THE SIZE RELATIONSHIPS SUBSISTING BETWEEN BODY LENGTH, LIMBS AND JAWS IN MAN

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INTRODUCTION

For the purpose of an investigation into the growth of nerves in man, measurements were made on a large number (*c.* 850) of male infants, children and adolescents living in London. The ages of the subjects ranged from 2 days to 20 years and their heights from 448 to 1870 mm. The examination of these data has revealed a number of facts of interest quite apart from those valuable for the original inquiry, and the present paper examines the relationships between the sizes of the limbs and jaw, and the total height of members of this group.

Many biologists have been interested in the relative sizes of parts of animals, and it has been maintained that the relationship between the size of a part and the size of the whole animal is best described by some kind of exponential (logarithmic) function. The outstanding examples of such work may be found in Huxley (1932) and Teissier (1937), while many other cases are described by Brody (1945). This kind of relationship is usually known as allometric or heterogonic. On the other hand, there is a considerable amount of evidence to show that in many cases the changes in size can be adequately described by a simple linear function, i.e. by stating that the size of the part is directly proportional to the total size of the animal.

MATERIAL AND METHODS

Since a wide range of ages had to be considered, four series of measurements were made. The first series was made on normal males of school-age attending London County Council schools in the north-west area of London. These boys were between the ages of 4 and 16 and were drawn from a uniform economic and social group. The second series was made on boys of 1–3 years. This series was carried out at day-nurseries in the same area from which the data covering the school-age boys were obtained, while the third series consisted of measurements made at University College Hospital on 50 newborn male infants, whose parents lived in similar areas. The fourth series of measurements was made on male University College students with an age range of 17–20. These students, although living in London at the time of the investigation, came from all parts of Great Britain and must have grown up in varied environments.

It will be seen that the whole series of these individuals may be considered to be a sample of the young male inhabitants of London, i.e. a sample from a highly cosmopolitan area.

The measurements were made in the following manner.

(*a*) *Height*

A 2 m. rule graduated in centimetres and millimetres, having metalled ends and placed vertically against a wall, door, etc., was used for measuring height. The subject stood with his back to this wall with heels, buttocks and occiput, touching the wall and eyes directed horizontally forwards.

(*b*) *Cubit*

A sliding caliper was used. In most cases the measurement was made on the right arm, exceptions being those cases in which there was a history, or other evidence, of injury to this arm. The subject was seated with his right forearm and outstretched hand placed prone on a table, the elbow being flexed to a right angle. The measurement was then made from the posterior surface of the elbow over the olecranon process, to the tip of the middle finger.

(*c*) '*Leg*'

The subject was seated with the right shoe removed and knee flexed to a right angle while the distance from the upper edge of the patella to the lower surface of the heel was measured with a sliding caliper. The word 'leg', as used in this paper, differs from standard anatomical usage in that the lower end of the femur, talus and os calcis are included.

(*d*) *Mandible*

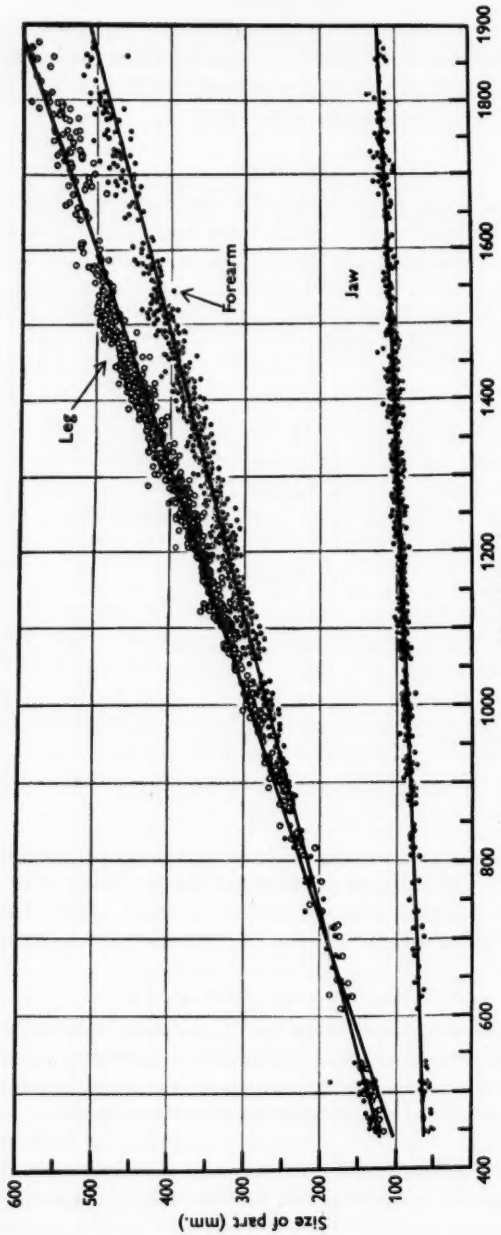
The measurement chosen as an index of mandibular size was the perpendicular distance between the bi-auricular axis and the symphysis menti. A 'head-spanner' was used for this measurement (see Pl. 1). This spanner consists of two adjustable wooden rods *A* and *B* with tapering ends attached to the frame as shown. The ends are made to fit snugly but not tightly into the external auditory meatus. A third adjustable rod *C*, calibrated so that direct readings can be made from it, makes contact with the symphysis menti and the perpendicular distance between the line *AB* and *C* is shown at *D* (on rod *C*).

Some variations in technique were used for measuring the infants. For height, the crown-heel length in the supine position was taken, measured by a large sliding caliper. Mandibular size was measured as before, and a small sliding caliper was used for the cubit and leg measurements.

ANALYSIS OF THE DATA

The data were plotted on squared paper and the result is reproduced in Text-fig. 1. It is obvious that some kind of linear hypothesis is suggested, since there appears to be no indication of curvature; moreover, there is no need to postulate any kind of polynomial or exponential relationship between the variables.

Denoting the values of the body height by x and those of the length of the part (limb or jaw) by y we may consider the linear regression of y on x. We assume that the x values are known accurately and that for any given x the corresponding values of y are normally distributed about a mean $a+bx$ with a standard deviation which must be estimated from the data. These assumptions are, of course, those



Text-fig. 1. Plotted points of all the observations and the regression lines calculated from the data.

usually made for fitting linear regressions; the calculations were made from ungrouped data. One set of data is less satisfactory than the other two since it is clear that any change in length of leg must lead to a change in total height and, consequently, there is a kind of spurious correlation between the measurements of leg and those of height. This, however, is unavoidable and the estimates of relative size are unaffected.

Table 1. *The values of the parameters to be inserted into the regression equation* $y = a + bx$. *The values of* $1{\cdot}96\sqrt{V}$ *are used to determine confidence limits as described in the text*

Part (y)	a	b	$\sqrt{V}$	$1{\cdot}96\sqrt{V}$
Arm	−0·30	0·27	8·975	17·59
Jaw	39·80	0·045	4·789	9·39
Leg	−50·31	0·34	14·430	28·28

Table 2. *The values of certain computed functions which present the information necessary for most statistical investigations on the data*

Part (y)	Number measured	Mean height ($\bar{x}$)	Mean y ($\bar{y}$)	Standard error of b	$\Sigma(x-\bar{x})^2$	$\Sigma(y-\bar{y})^2$	$\Sigma(x-\bar{x})(y-\bar{y})$	Correlation coefficient	Residual sum of squares
Arm	847	1246·77	335·89	0·001	75 864 940	5 584 438	20 457 214	0·99	68 069·244
Jaw	842	1249·07	95·69	0·0006	75 050 582	169 390	3 356 675	0·89	16 263·618
Leg	847	1246·77	379·35	0·0016	75 864 940	9 186 006	26 144 741	0·98	175 953·053

The individual measurements are not presented here from considerations of space, but they have been deposited and may be consulted at the Thane Library, University College, London. Summaries of the information derived from the data are shown in the Tables. Table 1 gives the values of certain parameters which will be discussed later, while Table 2 contains the results of certain computations which may be useful to other investigators.

It is quite clear from the values of the residual sums of squares that the regression coefficients are significant, and the high values of the correlation coefficients indicate the suitability of a linear hypothesis. Moreover, these coefficients show that the regression lines of y on x and x on y are practically coincident.

The lines have been calculated in the form

$$y = a + bx, \tag{1}$$

where y = length of part (mm.), x = height (mm.). If the parameter values are substituted into equation (1), we find the following equations for the regression lines:

(*a*) Arm/height $y = -0{\cdot}30 + 0{\cdot}27x$.

(*b*) Jaw/height $y = 39{\cdot}80 + 0{\cdot}045x$.

(*c*) Leg/height $y = -50{\cdot}31 + 0{\cdot}34x$.

The lines have been drawn to the plots of the data in Text-fig. 1.

The values for a have no biological significance since they are formed partly from means and partly from regression coefficients, but the values for b are measures of the change in lengths of the limbs and jaw for any given change in height. If the height increases by 10 cm. then the arm increases by 2·7 cm., the leg by 3·4 cm. and the jaw by only 0·45 cm.; the leg shows the largest relative increase, the arm a some-

what smaller increase, while the change in the length of jaw is very small indeed. In a certain sense, these values of b may be considered as measures of relative growth.

Furthermore, data of this kind provide norms which may make it possible to detect early stages of faulty skeletal development. At present, reliance has to be placed on subjective judgements, and the physician may have to wait some time before the skeletal defect is sufficiently obvious. With data of the kind used in this paper it is a simple matter to test if the skeletal development of an individual lies within the usual range of variation. The method to be used is outlined below, and a particular numerical case is worked out as an example.

The equations enable us to find the size which is to be expected for one or all of these parts for a given height. It is only necessary to substitute the value of the height for x in the appropriate equation and then the value of y (size of the part) is easily calculated. Such an estimate is of limited value since the equation has only been fitted to a sample of the population and no matter how large the sample size, any estimates of values of parameters for the whole population will be subject to error. It is possible, however, to specify boundaries within which the size of the part may be expected to lie. Such boundaries are frequently referred to as confidence intervals or confidence limits and were discussed by Neyman (1937); the theory of such intervals has also been explained by Wilks (1944) and Cramer (1946).

In order to find such confidence limits we must first specify the proportion of cases in which we hope our statement to be correct, e.g. 95 or 99%. Then we can calculate two numbers θ_1 and θ_2 for every x, such that if we make the statement that y lies between θ_1 and θ_2, we shall be correct in 95% (or 99%) of the cases in the long run.

The group of all the individuals who were measured comprises a sample drawn from the population of London inhabitants and the conclusions may be considered to be valid for this population providing that no values of height outside the range considered are involved. The conclusions would not necessarily apply to the inhabitants of another part of the country since the sample could not be assumed to be representative without further investigation.

If the sample upon which the estimates are based is small, the formulae for finding the confidence limits are a little more complicated,* but in the present case we may use the expression $Y = y \pm 1{\cdot}96\sqrt{V}$, where y is the value found from the regression equation and V is the estimate of variance found from the residual sum of squares. The values of $1{\cdot}96\sqrt{V}$ are given in Table 1.

By way of example, let us assume that we wish to know the length of arm to be expected for an individual 1747 mm. high. We wish to answer a question of the form 'Is it reasonable to expect a normal Londoner who is 1747 mm. high to have an arm 500 mm. long?' The regression equation for the arm is

$$y = -0{\cdot}30 + 0{\cdot}27x,$$

* The exact confidence limits for an individual are given by

$$Y = y \pm 1{\cdot}96\sqrt{V\left(1 + \frac{1}{n} + \frac{(x-\bar{x})^2}{\Sigma(x-\bar{x})^2}\right)}.$$

In the case considered the last two terms may be ignored.

and the substitution of 1747 for x in this expression leads to a value of 470·78 for y. The value for $1{\cdot}96\sqrt{V}$ given in Table 1 is 17·59 and the confidence limits for Y are

$$Y = 470{\cdot}78 \pm 17{\cdot}59,$$

and, with 95% confidence, we can assert that for a height of 1747 mm., the arm length lies between 453 and 488 mm. Consequently, we should consider an arm length of 500 mm. as 'abnormal' for this height.

DISCUSSION

The study of the relative growth of parts of organisms became more widespread after the publication of Huxley's book on *Problems of Relative Growth* (1932). The author showed that when the logarithms of the weights or dimensions of parts of an organism were plotted against those of the whole animal, the plotted points often appeared to fall about a straight line. If this were the case, it would imply an exponential or compound interest relationship between the variables of the form $y = bx^{\alpha}$; when the value of α differed from unity, such growth was described as heterogonic or allometric.

The results for many sets of data appeared to be convincing, but cases were frequently found when it was necessary to draw two or more straight lines in order to fit the logarithmic plots by straight lines. Moreover, certain observers, including D'Arcy Thompson (1942), found that a simple linear relationship was often adequate to describe the data, and since there is no *a priori* reason to prefer the allometric form and if the data show no curvilinear trend, then there is no justification for departing from an hypothesis of direct proportionality. The numerous theoretical objections to the allometric formula have been discussed in the essays by Reeve and Huxley and by Richards and Kavanagh in the volume edited by Le Gros Clark & Medawar (1945). The problems involved in fitting the allometric curve have been discussed elsewhere (Sholl, 1948).

It may be pointed out that it is sometimes thought that some justification for the allometry concept may be derived from the assumption that living cells themselves produce living cells which reproduce in their turn, i.e. a compound interest system. Reeve and Huxley point out that bone does not grow interstitially and there is little reason to assume that limbs would show this type of growth. There is a considerable literature on the subject and full bibliographies may be found in the essays cited.

The sets of data considered in this paper do not show any kind of allometric relation; the size of the part is directly proportional to the total size from the time of birth to maturity. It is true that, outside the range we have considered, e.g. in foetal life and during periods of life in which a more unrestricted type of growth prevailed, the heterogonic relationship might well hold. It is, however, interesting to note that during infancy and at puberty there is no evidence of such a tendency. Not only do the plotted points show no indication of any curvature but the high values of the correlation coefficients indicate that the linear hypothesis is justified; in fact, there is no reason to fit any kind of exponential curve. Although the sampling was not from a homogeneous group, the large number of observations with so small

a scatter indicates that the calculated lines do represent a mean tendency about which the values of the sizes of the part vary.

It is, of course, obvious that such lines give no measure of any growth rate, since such rates must be concerned with the change in size with respect to time. Time has not been considered in this investigation, and consequently, the gradients of the lines only give information as to change in size of the part in relation to change in size of the whole individual.

SUMMARY

1. Measurements of limbs, jaws, and total height were made on 795 male human beings from various parts of the London area. The ages ranged from the newly born (2 days old) to 20 years old, while the height ranged from 448 to 1870 mm.

2. The regression of the length of the part on the total body length is linear; consequently there is no evidence for allometric growth at any period between infancy and maturity.

3. Sufficient data are published and methods are given for finding the confidence limits between which the size of the part may be expected to lie when any particular value of the total length is considered. The application of this method as an aid to the diagnosis of pathological states is shown.

We wish to thank Prof. J. Z. Young for the advice and encouragement which he has freely given during the course of this work and we are most grateful to the London County Council for their co-operation in this work and, particularly, to Dr J. N. Dobbie, Senior Medical Officer of Health to the London County Council, and Dr G. W. Pritchett, the Medical Officer for the north-west region. We must also thank the Head Teachers of the various schools for their co-operation and Prof. F. J. Brown, lately Professor of Obstetrics in University College Hospital, for permission to make measurements on the newborn infants; Sister Edwards gave us expert assistance.

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SHEPHERD, SHOLL AND VIZOSO—SIZE RELATIONSHIPS OF BODY, LIMBS AND JAW

THE PROJECTION OF THE CAUDAL SEGMENTS OF THE SPINAL CORD TO THE LINGULA IN THE SPIDER MONKEY*

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INTRODUCTION

In investigating the degree to which the organization of the central nervous system is influenced by the presence of a prehensile tail in the spider monkey, we found that the eight caudal segments of the spinal cord which are exclusively concerned with the tail (Chang & Ruch, 1947*a*) are projected to a particular cell group of dorsal column nuclei (Chang & Ruch, 1947*b*) and a particular region in the thalamus (Chang & Ruch, 1947*c*). Most interesting of all is the finding reported below, i.e., that the projection of these terminal segments of the spinal cord is to the foremost part of the anterior lobe of the cerebellum.

This finding, we believe, offers anatomical support for the theory of topographical representation of the cerebellum as advanced in recent years by physiologists, namely that the tail, leg, arm and face are represented in the anterior lobe anteroposteriorly in that order.

EXPERIMENTAL RESULTS

Experiment 1. *Spider monkey series no.* 13; *adult male. Spinal transection at* Ca_1. *Operated* 1 *Sept.* 1944. *Sacrificed* 1 *Oct.* 1944. *Swank-Davenport preparation.*

A compact bundle of degenerated fibres is present in the periphery of the anterolateral funiculus just above the lesion. The ventral spinocerebellar tract is distinguished from the other components of Gowers' fasciculus by its comparatively small size, by the closer aggregation of the fibres, and by its ventrolateral position which is maintained throughout the cord.

In the lower medulla, the degenerated fibres in the ventrolateral fasciculus of Gowers can be followed to the region of the nucleus lateralis medullaris where they occupy a triangular area between the inferior olive and the descending trigeminal tract. Here, some of the fibres, mostly those of large size, veer medialward to the reticular formation dorsal to the inferior olive and the remaining fibres proceed farther cephalad. While ascending to the level of the caudal end of the pons, the cerebellar fibres turn dorsally and occupy the dorsolateral margin of the medulla, arching around the brachium conjunctivum. Some of them turn backwards, passing along the medial wall of the superior cerebellar peduncle and the others course through the anterior medullary velum to the cerebellum. In transverse sections

* This paper constitutes a part of a dissertation 'Segmentation, lamination and topographical projection of the nervous system with special reference to the tail of *Ateles*', presented in 1946 by H.-T. C. to the faculty of the Graduate School of Yale University in candidacy for the Degree of Doctor of Philosophy.

taken at the level of the exit of the vagus nerve, the spinocerebellar fibres appear in the region between the inferior olive and the descending trigeminal tract; and a few degenerated fibres are seen in the median plane of the cerebellum between the nuclei fastigii. The latter fibres are considered to be those passing to the uvula and pyramis.

In the sections taken at the level of the eighth nerve and also at the anterior end of the dentate nucleus of the cerebellum, the backward course of the spinocerebellar fibres along the medial border of the superior cerebellar peduncle is found. Here, the degenerated fibres are seen distributed along the median plane of the anterior lobe of the cerebellum.

From sections taken at the level of the pons, the dorsalward shifting of the ventral spinocerebellar tract and the termination of degenerated fibres in the lingula and the lobulus centralis are shown.

The degenerated fibres in the anterior lobe are strictly confined to the median plane.

Experiment 2. *Spider monkey series no.* 14; *adult, female. Left spinal hemisection at* Ca_1. *Operated on* 8 *Sept.* 1944. *Sacrificed* 26 *Sept.* 1944. *Swank-Davenport preparation.*

The degenerated fibres resulting from the left hemisection of the spinal cord at the first caudal segment are present in the fasciculus of Gowers on both sides, but those on the right side, which originate from cells at the site of the lesion on the left, are much fewer in number.

At the level of the pons, the cerebellar components of Gowers' fasciculus shift dorsally to the dorsolateral border of the brachium conjunctivum, curving around the dorsal aspect and then the medial aspect of the superior peduncle, and finally entering the cerebellum.

The distribution of the degenerated fibres in the cerebellum is similar to that seen in Exp. 1. In addition, the number of degenerated fibres to the left of the median plane of the anterior lobe is much greater than on the right side. This seems to be an indication of the predominant ipsilateral termination of the ventral spinocerebellar tract. The fibres cross in the great anterior cerebellar commissure. No degeneration products are found in the lateral extensions of the anterior lobe, in the paraflocculus, or in any other part of the cerebellar cortex.

Experiment 3. *Spider monkey series no.* 17; 1*-year-old female. Spinal transection at* Ca_1. *Operated on* 28 *Nov.* 1944. *Sacrificed* 11 *Dec.* 1944. *Swank-Davenport preparation.*

The medulla and the cerebellum were cut in sagittal sections in order to gain a better orientation of the degenerated fibres in the cerebellum. In the sections from the most lateral part of the medulla, the course of the degenerated fibres in the fasciculus of Gowers is distinctly stained. At the caudal end of the medulla, the degenerated fibres present a wavy appearance. They weave through the cell groups of the nucleus lateralis medullaris, which probably receive no terminations of the degenerated fibres. Farther cephalad, the degenerated fibres are grouped in a bundle passing over the dorsal surface of the inferior olive. From the caudal end of the pons onwards the tract gradually turns more dorsally. At a mid-pontine level the fibres are divided into two parts; the smaller ventral part proceeds farther forwards and

some of its fibres are gradually dispersed and probably terminate in the reticular formation. The large dorsal portion goes towards the floor of the fourth ventricle, beneath which the degenerated fibres are further divided into two subgroups. One group turns backwards to the cerebellum, chiefly by way of the anterior medullary velum and the other group, including the spinotectal and spinothalamic fibres, continues on to the midbrain and thalamus. The distribution of the degenerated fibres is shown in a sagittal section of the cerebellum (Pl. 1, fig. 1). Degenerated fibres coming from the anterior medullary velum are shown. Degeneration products are abundant in the lingula. The degeneration in the lobulus centralis (lobulus 2 of Bolk) is apparently heavier than in the lobulus 3 of Bolk. Only a few fibres enter the anterior margin of the large medullary lamina in the culmen. In the posterior part of the cerebellum, i.e., posterior to the fissura prima, only the upper and lower lips of the fissura secunda receive degenerated fibres from the ventral spinocerebellar tract (Pl. 1, fig. 2). The posterior part of the culmen, the lobus simplex, the declivus and the nodulus are virtually free of degeneration. All the degeneration products are confined to the white matter.

To summarize: In the spider monkey the ventral spinocerebellar tract can be found in the lowest segments of the spinal cord. In their course through the spinal cord and medulla, the fibres in this tract always occupy the most peripheral region and are immediately external to, and partly mingled with, the spinothalamic fibres. They enter the cerebellum by way of the anterior medullary velum and the anterodorsal aspect of the superior cerebellar peduncle. On reaching the cerebellum some fibres cross through the rostral part of the great anterior cerebellar commissure and both the crossed and uncrossed fibres run to the lingula, the lobulus centralis and the anterior folia of the culmen. No fibres go to the posterior culmen or to any part posterior to the fissura prima except an isolated bundle to the walls of the fissura secunda. After a transection of the spinal cord at Ca_1 the severest degeneration was found in the lingula and the anterior folia of the lobulus centralis; only a few fibres could be traced to the anterior part of the culmen and the walls of the fissura secunda.

DISCUSSION

In all of the three experiments described above, the lesions were made at the first caudal segments. Because the dorsal spinocerebellar tract in the caudal cord is not prominent and cannot be easily traced to the higher levels of the brain, the degenerations observed in the cerebellum are chiefly those contributed by the ventral spinocerebellar tract.

In 1909 McNalty & Horsley, working chiefly on macaques, concluded that fibres of the ventral spinocerebellar tract terminate in the whole anterior lobe of the cerebellum *except the lingula.* However, the termination of the ventral spinocerebellar tract in the lingula has been demonstrated since then by Ingvar (1918), and Beck (1927) in the cat; and Anderson (1943) in the rat. McNalty & Horsley's failure to find termination of spinocerebellar fibres in the lingula might be associated with the poorly developed caudal segments in the macaque.

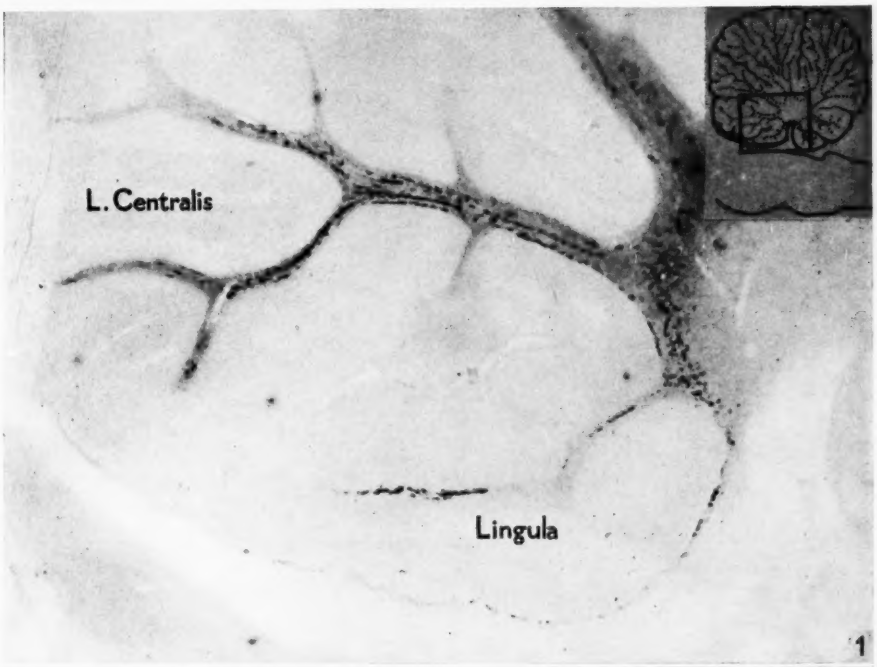
It is suggestive that such a large number of ventral spinocerebellar fibres should terminate in the lingula of the spider monkey, which has a very highly developed tail, and that the amount of degeneration in the lingula after a cervical anterolateral

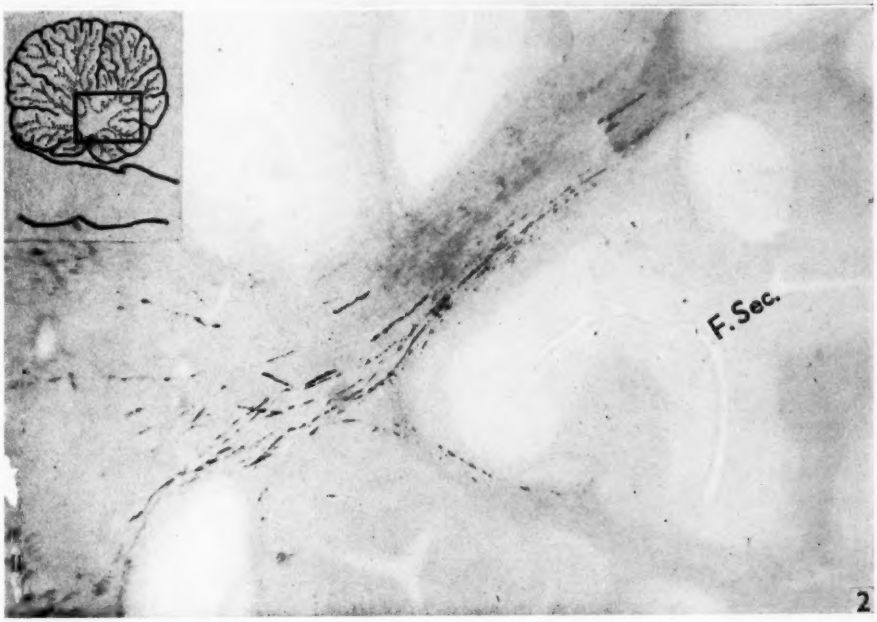
cordotomy is not significantly greater than that resulting from a caudal transection. It is evident that the lingula receives fibres mainly from the caudal segments of the cord which are largely related to the prehensile tail in this animal. Our findings afford an anatomical basis for the recent physiological findings of Adrian (1943), Snider & Stowell (1944), Dow & Anderson (1942), and Hampson, Harrison & Woolsey (1945). Adrian demonstrated by electrical methods that sensory impulses from the lower part of the body are projected to the rostral end of the anterior lobe of the cerebellum and those from the upper part of the body, to the posterior end. Snider & Stowell found that the somatotopic projection on to the cerebellum is in the same order as that postulated by Adrian. Hampson *et al.* described a sequence in the somatotopic localization of motor function in the cerebellum, with the tail end at the lingula. More recently (1946) they reported that the strongest motor effects in the tail were obtained by electrical stimulation of this particular region. It is, therefore, probable that both afferent and efferent cerebellar mechanisms for the tail are situated in the lingula. Another clue to the functional significance of the lingula is its special development in the sea mammals, in which the tail is a well-developed propulsive and 'steering' organ whereas the limbs are retrogressive.

The cerebellum of the spider monkey is characterized by a large and laterally expanded paraflocculus which Bolk (1906), from a morphological and phylogenetic point of view, related to the development of the tail in this animal. Bolk's correlation of the paraflocculus with the tail is probably correct, although the theoretical basis of his statement is no longer valid. He regarded the tail of the spider monkey as an organ of balance, functionally similar to the tail of whales. This is, however, not true. His second assumption that the paraflocculus is concerned with body equilibrium is unlikely, in view of the present knowledge of the cerebellum. According to the analysis by Larsell (1947), the paraflocculus belongs to the corpus cerebelli and is distinct ontogenetically from the flocculus. It has no fibre connexions with the vestibular apparatus. Thus, the explanation of the development and the bulk of the paraflocculus in tailed animals has to be found in a different direction. As first demonstrated by Ingvar (1918), the paraflocculus has afferent connexions from the spinal cord. In our material, the degenerated fibres could not be followed as far as the paraflocculus, but could be traced to the upper and lower lips of the fissura secunda, i.e., to the inferior part of the pyramis and the superior part of the uvula. It should be noted in our Pl. 1, fig. 2 that the degenerated fibres are sharply confined to the lower half of the medullary lamina of the pyramis and the upper half of the arbor vitae of the uvula. This distribution of the afferent fibres from the caudal region of the cord is perhaps a clue to the function of the paraflocculus, since according to Larsell & Dow (1935) the paraflocculus is derived from the pyramis and uvula.

SUMMARY

The degenerated ventral spinocerebellar fibres, after spinal transection or hemisection at Ca_1 in three spider monkeys, have been traced to the anterior part of the anteric lobe of the cerebellum, with the heaviest degeneration in the lingula and the anterior folia of the lobulus centralis. No degenerated fibres go to the posterior folia of the culmen or to any part posterior to the fissura prima except a few fibres to the walls of the fissura secunda.





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EXPLANATION OF PLATE

Fig. 1. Photomicrograph from a sagittal section of the cerebellum, illustrating the fibre degeneration in the lingula and the anterior folia of the lobulus centralis 13 days after spinal transection at Ca_1. The square in the inset figure indicates the location of the section photographed. Spider monkey no. 17.

Fig. 2. Photomicrograph from a sagittal section of the cerebellum, showing the distribution of degenerated fibres in the medullary laminae above and below the fissura secunda. Spider monkey no. 17.

TWO EARLY HUMAN EMBRYOS

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The two embryos described here were given to me by Prof. J. H. Biggart, who had recovered them from curettage material removed by Prof. C. H. G. Macafee; I have named the older specimen the Biggart Ovum and the younger the Macafee Ovum. The material was fixed in formol-saline and later transferred to formol-Zenker; it was embedded in paraffin, cut at 6μ and stained with haematoxylin and eosin.

THE BIGGART OVUM

The Biggart ovum was recovered from a woman aged 24 years; married 7 years; one child 4 years 8 months; no miscarriages; menstruation began at 15, 7-day loss recurring very regularly every 21 days; last menstruation stated to have ended 29 June; admitted to hospital 12 July and curetted 16 July; coitus said to have occurred only once between the end of menstruation and admission, probably on 2 July and certainly between 1 and 5 July.

The specimen consists of a piece of endometrium in which the ovum is very superficially implanted (Pl. 1, fig. 1). Examination shows the preservation to be good and that only some peripheral villous material and one section containing embryonic material proper are missing; but some of the sections are a little distorted. It was found to be cut almost vertically to the embryonic shield and obliquely at 13° to its sagittal plane. The primary measurements in millimetres of the ovum and its parts are as follows.

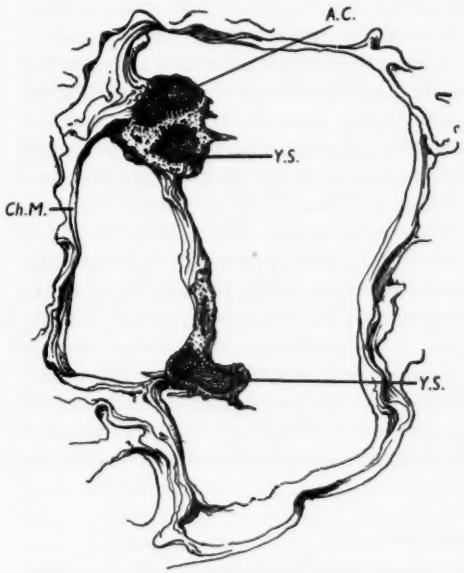
	Antero-posterior	Vertical	Transverse
Trophoblast, external, including the villi	2·53	2·10	1·68
Trophoblast cavity	1·53	1·05	0·83
Shield ectoderm	0·27*	0·027	0·16
Amniotic cavity	0·22	0·10	0·16
Yolk-sac cavity	0·19	0·82	0·18

* Taken diagonally from the graphic reconstruction.

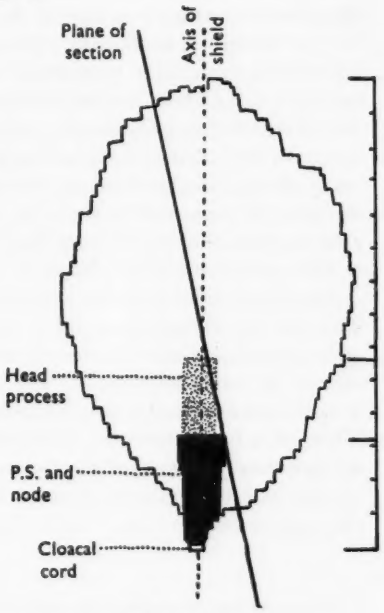
The endometrium shows early decidual reaction and contains peripheral syncytium beyond the limits of the trophoblastic shell; there is no zone of necrosis. The glands are dilated and in the saw-tooth phase; they contain extravasated blood and secretion. Large blood sinuses are present beneath the ovum and seem to have limited the villus formation over one part of the vesicle. The site of entry is wide and filled with the thin ab-embryonic wall of the chorionic vesicle covered by a thin layer of schlusscoagulum; there is no operculum.

The trophoblast, which is lined with a chorionic mesoderm, consists of cytotrophoblast and syncytiotrophoblast, and villi are present over most of it, but are absent over the superficial surface and over one part of the deep surface near a large venous sinus (Pl. 1, fig. 1). Many of the villi contain a mesodermal core and some have begun to branch.

The chorionic vesicle contains a granular coagulum surrounding the embryonic rudiment, which consists of an embryonic shield, a small amniotic cavity, and a larger yolk-sac which is continued by a long narrow tubular duct into a large irregular expansion (Text-fig. 1). The embryonic rudiment is joined to the chorionic mesoderm first by a broad connecting stalk attached to the amnion, and secondly by mesoderm at the distal expansion of the yolk-sac (Text-fig. 1).



Text-fig. 1.



Text-fig. 2.

Text-fig. 1. Drawing of wax model cut to show the relations of the embryonic mass. *A.C.* Amniotic cavity. *Ch.M.* Chorionic mesoderm. *Y.S.* Yolk-sac.

Text-fig. 2. Scale diagram of surface view of the embryonic shield.

The embryonic shield is a broad oval in dorsal view and is not quite symmetrical about its long axis (Text-fig. 2). It is slightly convex towards the amniotic cavity. Its margins are continuous with the thinner amniotic ectoderm, the transition being clearly marked (Pl. 2, fig. 4). The shield ectoderm consists of high columnar cells which in places appear to be stratified, but this is due to the obliquity of the section; those of the central area are larger than those nearer the margin and are more vacuolated and granular. At the posterior end of the shield and reaching its margin there is a median linear strip of lighter more loosely arranged polyhedral cells (Pl. 2, fig. 5); the strip extends over six sections and as measured by reconstruction is 0·059 mm. long and 0·036 mm. broad, that is, it is a little more than one-fifth of the length of the shield (Text-fig. 2). The cells forming it are continuous in front and at the sides with the general shield ectoderm, and below they are in close contact with the gut endoderm but can be distinguished from it; there can be little doubt that it is the primitive node and streak.

The main interest of the specimen lies in the condition of the yolk-sac. The form of its three parts is shown in Text-fig. 1. The wall of the whole of the proximal part consists of a single layer of round or oval cells much smaller than the overlying ectodermal cells and easily distinguishable from them. Cells of the same kind bound a small wide diverticulum which passes backwards, below the level of the connecting stalk, under the posterior end of the shield, which may represent the allantoic diverticulum or may be a part of the expanding yolk-sac which is later incorporated in it as described by Florian (1930). The cells lining the duct part of the sac are cuboidal in form, have large round nuclei and are arranged in a single continuous layer (Pl. 1, fig. 2); on the whole they are slightly larger than the cells of the proximal part. The cells lining the distal expansion (Pl. 1, fig. 3) gradually change from cuboidal above to thin flattened squamous-like cells below, and over the greater part are easily distinguishable from the covering layer of mesoderm; but the central part of the ab-embryonic wall is very thin and consists of a single layer of very flattened cells the nature of which, endoderm or mesoderm, I am unable to decide.

The mesoderm covers the duct and the proximal part of the yolk-sac and is a continuous layer over the amnion; it forms the broad irregular connecting stalk. There is a small amount of intra-embryonic mesoderm in the peripheral rim of the embryonic shield which is directly continuous with the extra-embryonic mesoderm; and in the angle between the anterior end of the shield and the yolk-sac there is a more loosely arranged mass of mesoderm which may well be the protocardiac area. There are a few projections, with indications of cavity formation, in the mesoderm of the proximal yolk-sac and cavities in the chorionic mesoderm, especially in the region of the connecting stalk (Pl. 2, figs. 4, 5); these have been taken to be the first evidences of angiogenesis.

Discussion

The next menstruation, from the history given, should have begun on 14 July, and, taking ovulation to have occurred 14 ± 2 days before this, the single coitus probably on 2 July, falls within the probable period of ovulation 28 June to 2 July; the probable coital age is thus $13\frac{1}{2}$ days. The general dimensions, structure and differentiation of the embryo, and the appearances of the decidua agree with this estimate and place the ovum in Streeter's (1942) Group VII; and in comparison with known ova it appears to be more developed than embryo No. 7801 ($13\frac{1}{2}$ days) of Heuser, Rock and Hertig (1945), of about the same stage as the Yale embryo (14 days) (Ramsey, 1938), and younger than the Falkiner ovum (15–17 days) (Martin & Falkiner, 1938). The interest lies in the hitherto undescribed form of the yolk-sac.

The presence of a duct-like process leading distally from the yolk-sac and connected to the chorionic mesoderm was described by Bryce (1924) in the ovum T.B. 2 and he noted that similar formations were present in other ova (Schlagenhaufer and Verocay, Fetzer, Strahl-Benecke) and that in the Frassi ovum there is a detached vesicle lined with endoderm which is connected to the yolk-sac by mesoderm. Heuser *et al.* (1945) describe in embryo No. 7801 several large vesicles in the peripheral part of the chorionic cavity, the walls of which are for the most part composed of a single layer of flat squamous-like cells, but occasionally containing patches of cuboidal cells; the largest vesicle is attached by a faint strand of cytoplasm, which itself contains

a vacuolated group of cells, to the yolk-sac. The same authors also describe in embryo No. 7802 (16½ days) a tapering funnel-shaped ventral process of the yolk-sac the mesodermal layer of which can be traced, as a fine filament, across the chorionic cavity to the mesoderm of the opposite side. Heuser & Wislocki (1935) have shown in the sloth (*Bradypus griseus*) that in early stages there is a chorionic attachment of the ab-embryonic wall of the yolk-sac and the formation of two yolk-sac vesicles connected, for a time, by a narrow duct. Later the connecting duct disappears, leaving a definitive yolk-sac under the shield and an endoderm-lined cyst-like cavity attached to the ab-embryonic wall of the chorion. Martin & Falkiner (1938) state that the yolk-sac of the Falkiner ovum is partially divided into two by a septum; and that the endoderm lining the cavity of the yolk-sac dorsal to the septum is cuboidal, and the endoderm of the part ventral to it is thin, flattened and smooth, except at the extreme ventral pole where it also is cuboidal. They also give details of a similar partial subdivision of the yolk-sac in a 15-day-old macaque embryo (No. C 571) and state that the differences of the two types of endoderm are more evident than in the human. They offer two explanations for the occurrence of the divisions of the yolk-sac. They state first (p. 257) that the grooves (external to parts of the septum) probably represent the earliest sign of the folding-off of the embryo, and that they separate the gut endoderm dorsally from the yolk-sac vesicle endoderm ventrally, as suggested by Streeter. On p. 266 they state that if the process of taking up evaginations of the yolk-sac 'had gone a little further the septum partially dividing this region of the yolk-sac would have disappeared and the part of the allantois situated in the caudal wall of the yolk-sac' (i.e. *below the septum*) 'would have been incorporated in the yolk-sac cavity'. This implies that some of the thin flattened endoderm assigned earlier to the yolk-sac vesicle becomes transformed into cuboidal gut endoderm. It is apparent, however, from a study of the Falkiner ovum, that the partial division of the yolk-sac seen in it is of a different nature from that seen in the present specimen. The endoderm cells lining the duct part of the yolk-sac of the Biggart ovum are slightly larger than the cells lying below the shield ectoderm and lining the proximal part of the yolk-sac cavity. It is only in the distal expansion of the yolk-sac that the lining cells become thin, flat and smooth, and in part of the ab-embryonic wall they are even more flattened and are either endoderm alone or mesoderm (Pl. 1, fig. 3). The appearances in the Biggart ovum are much more suggestive of the condition found in the 12-somite sloth. This stage could easily lead to the condition found in the 13½-day human (No. 7801) by Heuser *et al.* or to that in the 38-somite sloth embryo. The appearance of the endoderm cells of the ab-embryonic parts of the vesicle possibly is due to a slow rate or absence of division of the cells, or perhaps to a falling off of the activity gradient and to relatively poorer nutrition at the ab-embryonic region. The 'stretching-out' of the yolk-sac by the expansion of the whole chorionic vesicle, to which the yolk-sac is attached, probably is the direct cause of the thinning of the distal yolk-sac cells, the duct cells remaining, or becoming, cuboidal because of the small diameter of the duct relative to the rest of the yolk-sac.

The origin of the endoderm cells lining the yolk-sac still remains uncertain. The spread round of the endoderm cells from the periphery of the embryonic endoderm as seen in the macaque yolk-sac, must, in the human, be either very rapid or altogether omitted. If the spreading of endoderm cells round the interior of the exo-coelomic

membrane is absent or only partial, then the yolk-sac cells, as distinct from the endoderm cells below the shield ectoderm, must arise by delamination from the precocious mesoderm forming the exo-coelomic membrane as is stated to be the case by Streeter (1937). The absence of a definite double layer of cells over the ab-embryonic pole of the yolk-sac may suggest a migration of endoderm cells from the original embryonic endoderm cells, the migration not having been completed; the single layer present would then be described as mesoderm. But, alternatively, the delamination process may have been delayed over the less well-nourished ab-embryonic region or the second layer of cells may have already disappeared. It is unfortunate that the histological appearances are such that one cannot be certain whether this layer belongs to endoderm or mesoderm.

Whatever the exact mode of origin of the endoderm cells may be, it is evident that this ovum illustrates a stage in the formation of the human definitive yolk-sac intermediate between that described by Hertig & Rock (1941) for ovum No. 7700 (12½ days) and that by Brewer (1938) for the 15-day Edwards-Jones-Brewer embryo. Also that this stage, which has not previously been described for man, resembles the similar stage in the 12-somite sloth, and closely resembles the condition postulated by Hamilton, Boyd & Mossman (1945) in their scheme for the development of the yolk-sac.

THE MACAFEE OVUM

The Macafee ovum was recovered from a woman aged 26 years; her last menstruation was from 20 to 26 January; coitus occurred on 22, 25 and 30 January; she was admitted to hospital on 3 February and was curetted on 5 February; the expected date of her next menstruation was 11 February.

The material obtained from the curetting was cut into a diagnostic section and fifty-one serial sections separated from the former by a small interval due to loss of sections. The diagnostic section, of which Pl. 2, fig. 6 is a photograph, is nearest the centre of the ovum, and shows an endometrium well prepared for implantation. The endometrium is not grossly oedematous and there is no marked leucocytosis and no large haemorrhagic areas. Some capillary sinusoids were found to communicate with the spaces in the outer part of the trophoblast. The glands are dilated, but do not contain blood or pent-up secretion; some of them are being engulfed by the syncytiotrophoblast and their cells are disintegrating in the trophoblast spaces. There is no necrotic zone, and no syncytial masses of peripheral trophoblast were seen.

The ovum, which from the wax-plate reconstruction is seen to be ovoid in form, is very superficially embedded so that a large part of it is exposed and uncovered by uterine tissue. The measurements of the largest section are: trophoblast, maximum external $1{\cdot}13 \times 0{\cdot}63$ mm. and trophoblast cavity $0{\cdot}58 \times 0{\cdot}33$ mm. The trophoblast wall consists of two layers, an inner of large cuboidal cells lining the cavity, and projecting outwards as cell columns, and an outer thin covering layer of syncytium. This syncytium bounds lacunar-like spaces and peripherally forms a continuous layer which constitutes the junctional zone with the maternal stroma. There is a general absence of activity penetrating syncytium from this layer and the line of demarcation between it and the uterine stroma is quite definite. The trophoblast spaces contain

a coagulum and only a small amount of maternal blood cells. The trophoblast is lined internally with a layer of extra-embryonic mesoderm which does not indent the trophoblast columns. In the section nearest the centre of the ovum (Pl. 2, fig. 6) the mesoderm forms what appears to be an exo-coelomic membrane, but this cannot be traced in the more peripheral sections; there the mesoderm is smaller in amount and forms a much thinner layer. There is no embryonic rudiment.

Discussion

The superficial implantation and the general histological appearance of the specimen indicate that the ovum was probably dying and being cast off by the maternal tissues. The coital and menstrual histories are believed to be reliable, and assuming that the coitus on 25 January was the fertile one, give to the ovum a maximum coital age of $10\frac{1}{2}$ days. The measurements of this ovum, as compared with other pre-villous ova, notably the Hertig & Rock ova (Carnegie Nos. 7699, 7700, 7950 and 8020), the Davies-Harding, Barnes, Miller, Dible and West, Kleinhans, Werner, Scipiades and Marchetti ova, also place it between 9 and 13 days of age. In appearance it closely resembles that of Carnegie No. 7950 (Rock & Hertig, 1942). The engulfment and disintegration of the uterine glands by the syncytium are unusual in an ovum of this early stage of development and indicate that the invasive power of the syncytium must, at one time, have been considerable.

SUMMARY

Two human ova recovered from uterine scrapings are described. The older specimen (14 days) is normal, but is rather superficially embedded. It shows a large yolk-sac with proximal and distal dilatations connected by a narrow tubular duct. The proximal and duct parts are lined with cuboidal cells. The distal expansion is partially lined with flattened endoderm cells and also shows an area one cell in thickness over the ab-embryonic pole. The significance of the appearance of the yolk-sac and the general position of the ovum with regard to other early human ova are discussed.

The younger ovum (10 days) is incomplete. It is superficially embedded, and probably pathological. Erosion and engulfment of the uterine glands have occurred at an earlier stage than is usually described in the human. Its probable age and its general chronological position are discussed.

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EXPLANATION OF PLATES

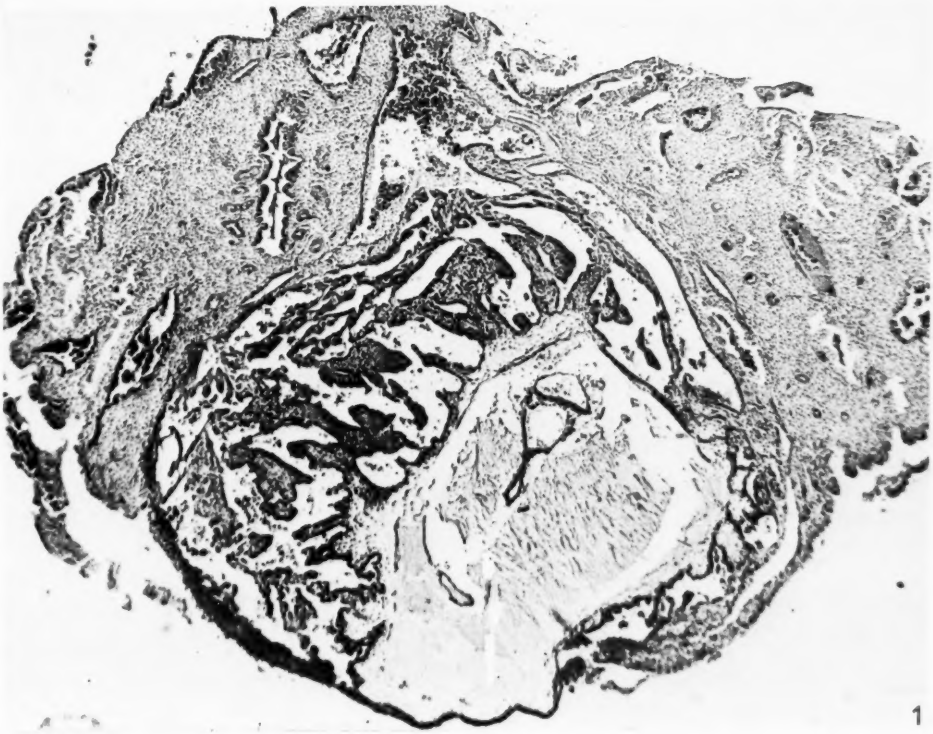
Figs. 1–5 are of the Biggart ovum, fig. 6 of the Macafee ovum.

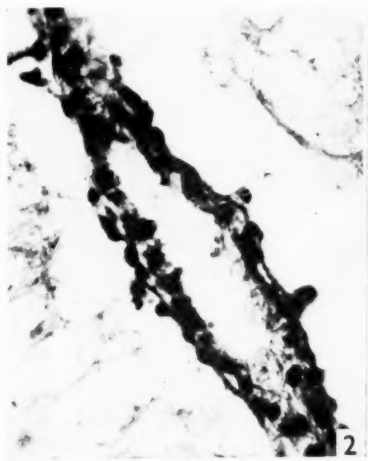
PLATE 1

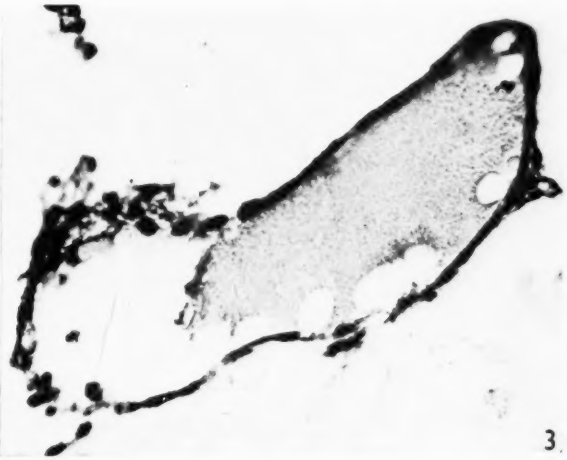
Fig. 1. The ovum *in situ*. Sect. 81. ×38.
Fig. 2. The duct part of the yolk-sac. The distal end is inferior. Sect. 77. ×375.
Fig. 3. The distal expansion of the yolk-sac. The end of the duct just appears above, on the left. Sect. 77. ×375.

PLATE 2

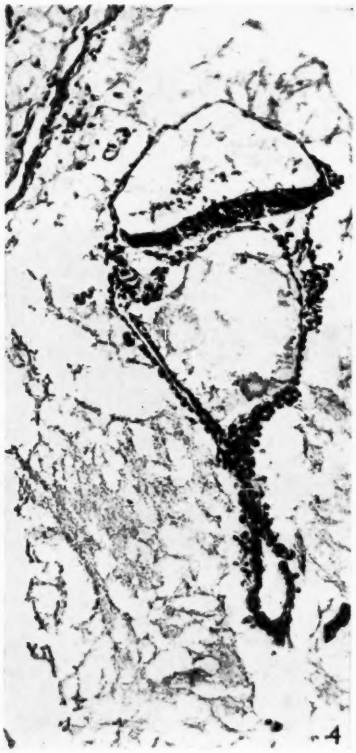
Fig. 4. The embryonic rudiment. The distal expansion of the yolk-sac is not figured. Sect. 81. ×140.
Fig. 5. The opening of the duct part into the distal expansion of the yolk-sac which shows its thin ab-embryonic wall below. Sect. 75. ×95.
Fig. 6. The Macafee ovum *in situ*. Diagnostic section. ×65.

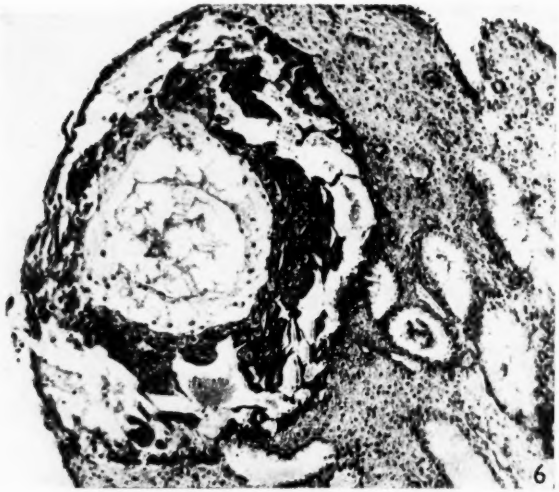






MORTON—TWO EARLY HUMAN EMBRYOS





MORTON—TWO EARLY HUMAN EMBRYOS

OVARIAN AUTOGRAFTS IN MONKEYS

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Four main and conflicting conclusions emerge from the extensive literature on the origin and development of ova in vertebrates (Heys, 1931; Everett, 1945). The first is that the parent cells of the ova are segregated very early from the soma, and migrate in man from the yolk-sac (Witschi, 1948) to the genital ridge where they establish themselves in the germinal epithelium. The second combines this view with the assumption that elements of the germinal epithelium derived from the mesothelium of the genital ridges also produce germ cells. The third presupposes an early and extragenital segregation of primordial germ cells, but holds that these all degenerate, and that in the differentiated gonad only the germinal epithelium produces ova. The fourth denies any early segregation of germ cells, and attributes to the germinal epithelium the total potency of the body to produce germ cells.

The unsettled nature of the problem is further exemplified by the literature on the formation of germ cells after puberty. Here there are three main views. The first is that all the ova that are going to appear in the life cycle of an individual do so before puberty. The second is that the germinal epithelium continues to produce germ cells throughout reproductive life, and in phase with the oestrous or menstrual cycle. The third is that not only the germinal epithelium but also other, as yet unidentified, cellular constituents of the ovary produce ova.

Discussion of these various matters has usually followed hard and fast lines, in the sense that most authors have regarded one conclusion about the origin and formation of the germ cells as being incompatible with any other. The evidence also clearly demonstrates the great difficulty of using purely histological observations of normal material as a basis for conclusions about the temporal phases of dynamic processes. Indeed, the issues have become so involved and confused that it would seem unlikely for any of the conflicts to be resolved except by the study of purely experimental material.

One piece of evidence that has been adduced in favour of the view that the germinal epithelium is not essential to the formation of germ cells is the presence of ova in ovaries from which it has disappeared. In an extensive series of experiments on the rat, guinea-pig, cat, and opossum, Moore & Wang (1947) have shown that corpora lutea and active follicles in all stages of development are to be found a year after the germinal epithelium has been eliminated by the application of salicylic acid to the ovarian surface. Follicles in varying stages of development have also been reported in ovarian grafts whose germinal epithelium has been completely replaced by fibrous tissue (e.g., Marshall & Jolly, 1907, 1908; Pettinari, 1928; Breward & Zuckerman, 1949). In so far as it is impossible to differentiate between the persistence of pre-existing follicular elements and the neo-formation of ova from unidentified cells in the ovarian substance, none of this evidence is in itself conclusive; it is, however, useful to the solution of the general problem.

It is for that reason that the following data relating to monkey tissue have been assembled in the present paper.

The transplantation of ovaries in monkeys was first reported by Marshall & Jolly (1908). These workers grafted the ovaries of an unspecified type of monkey on to the peritoneum of another unspecified monkey. At the same time, they removed the host's ovaries and transplanted them also on to the peritoneum. When it was killed 2 months later, the host's own ovaries were still in their new position, although they had 'undergone a certain amount of fibrous degeneration', whereas the homograft ovaries had been completely absorbed. The heterotypic grafting of monkey and ape ovaries into human beings achieved great notoriety in association with the name of Voronoff. Hannan (1931) has attempted the reverse procedure and has grafted human ovaries into rhesus monkeys, but without success. None of the voluminous literature on ovarian grafts in Primates contains any quantitative appreciation of the results or of the histological changes.

MATERIAL AND METHODS

The tissues whose histological study forms the basis of the present report were taken from two adult rhesus monkeys (*Macacus mulatta*) which were used in experiments (in 1933 and 1934) designed to disclose a possible neural factor in the control of menstrual bleeding (Zuckerman, 1938). An account of the experiments has not been published hitherto, and is therefore given here.

Experiment 1 (*Mm.* 77)

The animal, which weighed 5·3 kg., was operated upon on the first day of a menstrual cycle. After being anaesthetized with nembutal, given intraperitoneally, the abdomen was opened, and the ovaries removed. A number of small follicles, none bigger than 2 mm. were visible on the surface of the right ovary, and on its supero-medial aspect was an old corpus luteum, with a surface scar. The left ovary showed the stigma of an apparently very recent corpus luteum. The ovaries were transplanted into the eyes. The lids were retracted at the lateral canthus, and after trimming at the hilum and both poles, the ovaries were transplanted whole into the posterior chambers of the eyes. There was some escape of vitreous humour, but very little bleeding. Tenon's capsule but not the sclera was stitched.

The animal rapidly recovered from the operation and retained effective vision. She died 10 months after the transplantation.

The eyes were fixed in formalin, serially sectioned at 20μ and stained with Meyer's haemalum and eosin.

In the 10 months after the first operation, a study was made of the effects of interruption of sympathetic impulses along the cervical sympathetic chains and spinal cord. Uterine bleeding occurred for the first time 158 days after the transplantation of the ovaries. The animal then experienced three menstrual cycles lasting 44, 45 and 42 days respectively. She died on the sixteenth day of the succeeding cycle, her weight at death being 5·1 kg.

Histological study.

Every section was examined, and all follicles the ova of which showed a nucleolus were counted. They were classified into six types (see below), and differentiated as normal or atretic. This classification is similar to the one previously used by Green & Zuckerman (1947).

Experiment II (*Mm.* 88)

This adolescent rhesus monkey weighed 4 kg. when operated on. The transplantation was carried out in the same way as in the first experiment, except that the sclera as well as Tenon's capsule was sutured. She made an uneventful recovery, but her vision was much impaired by the operation.

The animal died 11 months later. During this interval she was given four courses of gonadotrophin injections, and a study was made of the effects on the menstrual cycle of general vaso-motor shock and of interruption of sympathetic impulses in the cervical chains.

Four phases of uterine bleeding occurred during the 11 months after the ovaries were transplanted.

At autopsy no ovarian tissue was found in relation to the Fallopian tubes of either animal.

RESULTS

Mm. 77

Left ovary. The ovary had fixed itself to the posterior surface of the lens, its lateral extremity on the attachment of the suspensory ligament. The retina in many places showed pathological change.

The maximum dimensions of defined ovarian tissue are 7·5 mm. long, 2·65 mm. wide and 1·5 mm. thick. The tissue is well vascularized and compact, and is covered behind by flattened cells different in appearance from normal germinal epithelium. No epithelium covers the anterior surface, which is attached to the capsule of the lens.

The ovarian stroma contains many epithelial elements. Healthy ova are absent, but there are the remains of a few primordial follicles undergoing atresia. There are many follicular cysts, the largest of which is irregular in shape and 0·7 mm. in diameter. A large corpus luteum is present.

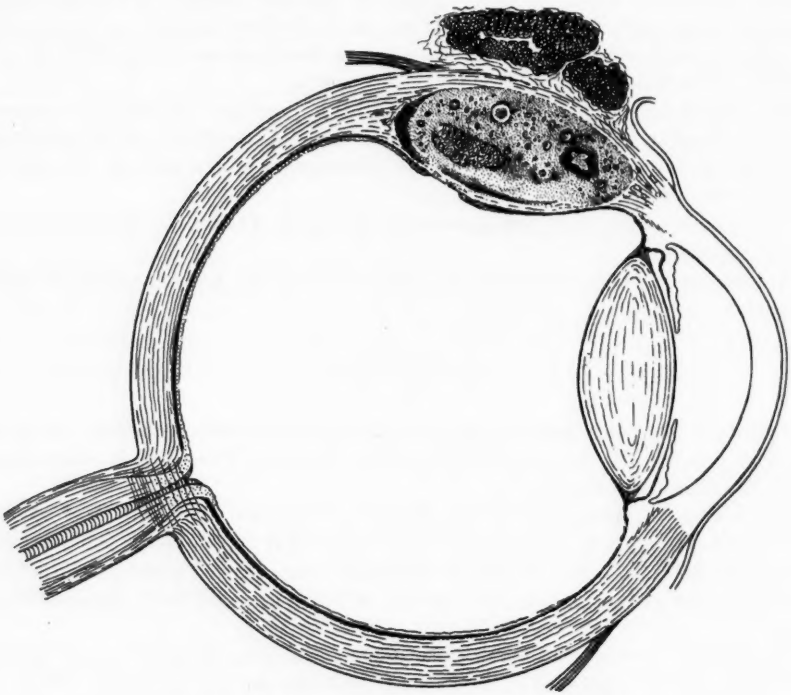
Right ovary. The ovary implanted itself into the lateral part of the eyeball within the sclera, its anterior pole abutting on the lachrymal gland. The ovary is compact and had retained a more normal ovarian appearance than its fellow (Text-fig. 1). Its maximum dimensions were $8{\cdot}8 \times 5 \times 3$ mm.

The retina of this eye appeared to be healthy, and the lens was not occluded.

The ovary contained three distinct, and two ill-defined corpora lutea, as well as many follicles (Pl. 1, fig. 1). The largest follicle measures $2{\cdot}5 \times 2{\cdot}2$ mm. in the stained preparations. The two most recent corpora lutea (Pl. 1, fig. 2) are situated in the half of the ovary adjacent to the lachrymal gland, and are separated from each other by a large atretic follicle. There is no sign of luteinization in any of the larger follicles, nor of any germinal epithelium.

The follicles were classified as follows:

Type 1: single layer of flattened or rounded granulosa cells.
Type 2: single layer of cuboidal granulosa cells.
Type 3: two layers of cuboidal granulosa cells.
Type 4: three layers of cuboidal granulosa cells.
Type 5: more than three layers, but no antrum.
Type 6: antrum present.



Text-fig. 1. Schematic diagram of right ovary of Mm. 77.

The total number in the ovary were:

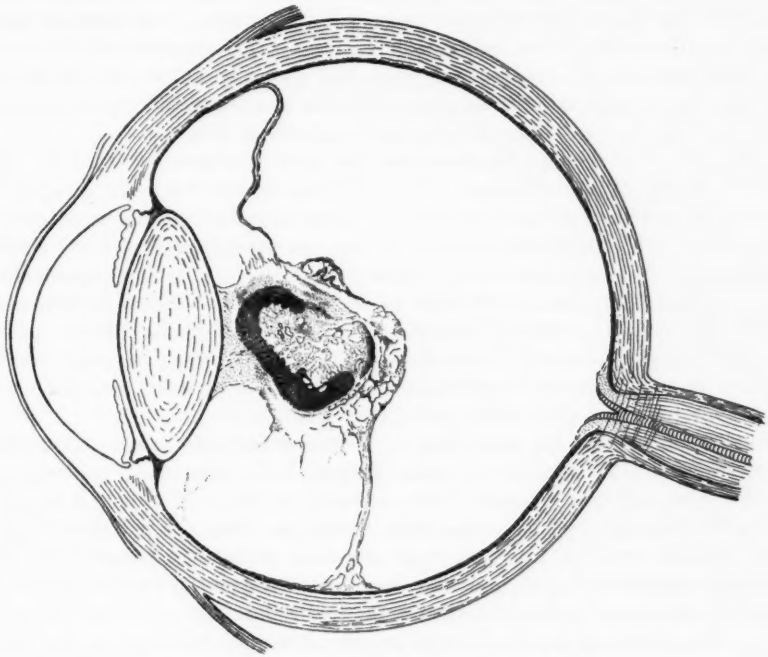
	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Total
Normal	121	6	6	2	4	—	139
Atretic	145	29	24	6	7	1	212
Total	266	35	30	8	11	1	351

Mm. 88

Left ovary. The ovary had implanted itself into the base of the ciliary body, which was completely disorganized. It measured $2{\cdot}5 \times 2{\cdot}4 \times 1$ mm., and was not as compact as its fellow. In places the cortical zone appears to be incomplete. There was no sign of germinal epithelium.

No healthy follicles containing ova were found. There were many degenerating follicles enclosing hyaline plaques, whose indistinct outlines were reminiscent of a degenerating zona pellucida. Some follicles had a vascular core, others seemed to be solid balls or cylinders of granulosa cells.

Right ovary. The ovary has a typical ovoid form and except at one point is embedded completely in a mass of new tissue which stretches obliquely between the lens in front and the posterior wall of the vitreous behind (Text-fig. 2). The choroidal



Text-fig. 2. Schematic diagram of right ovary of Mm. 88.

attachment of this mass of tissue is in the form of a prismatic pedicle. The maximum dimensions of the ovary in the stained preparations are $3{\cdot}75 \times 2 \times 1{\cdot}8$ mm. It consists of a core of vessels and fibrotic atretic follicles, which have undergone hyaline change, surrounded by dense chromophil cortex consisting of closely packed stromal cells and ovarian epithelial elements. The maximum diameter of the cortical zone in the stained preparation is about 0·2 mm. Germinal epithelium was not recognizable.

Within the dense ovarian cortex can be recognized aggregates of granulosa cells and cornified plaques of the kind observed in the left ovary. There is only one large follicular cyst, irregular in shape, 0·6 mm. in diameter. No healthy or atretic follicles with nucleus and nucleolus at any stage of development could be seen, and the appearances suggested a greatly enhanced rate of follicular atresia.

Uterine bleeding occurred in this animal, in spite of the absence of all cyclic

structures in the ovary. This observation can be correlated with the fact that oestrous cycles continue in mice whose ovaries after X-ray sterilization no longer exhibit any cyclic changes (Parkes, 1926; 1927 *a*, *b*, and *c*).

DISCUSSION

The view generally held by those who attribute the development of ova entirely to the germinal epithelium is that the germ cells have a short life cycle which is correlated with the length of the oestrous or menstrual cycle (Allen, 1923; Evans & Swezy, 1931). The very high rate of atresia that is always to be observed in the ovaries of mammals undoubtedly supports this view, and makes it difficult to suppose that follicular elements observed in an ovary a year or so after the disappearance of the germinal epithelium can represent the survival of pre-existing ova.

Unless ova have a long and not a short life cycle, it is impossible not to accept Moore & Wang's (1947) view, based as it is on observations of the ovaries of the rat, guinea-pig, cat and opossum, a year after the germinal epithelium had been destroyed with salicylic acid, that the presence of an active germinal epithelium is not essential for oogenesis. This view obtains some support from the tissue-culture experiments of Martinovitch (1938). These showed that oocytes are formed after most of the germinal epithelium cells have emigrated into the culture medium. As the cultures survived for only 1 month, however, it may be, as Martinovitch himself suggests, that this evidence should not be taken to indicate more than that the germinal epithelium is not essential to the growth of the ovum after synapsis.

Further support for the view that the germinal epithelium is not essential is, however, afforded by observations which suggest that ovarian tissue can regenerate in mice after complete removal of the ovaries (e.g. Parkes, Fielding & Brambell, 1927). On the other hand, the work of Heys (1931) and Pincus (1936), which suggests that 'complete removal' in a mature rat or mouse is almost impossible because of the folding and lobulation of the ovary, casts some doubt on this line of evidence. The conclusion is, however, also supported by the fact that ova are not formed in a mouse ovary after sterilization with X-rays, in spite of the persistence of an apparently normal germinal epithelium (Parkes, 1926, 1927 *a*, *b*, and *c*; Brambell & Parkes, 1927; Brambell, Parkes & Fielding, 1927 *a*, *b*; Parkes, Fielding & Brambell, 1927). Here, on the other hand, it can be argued (Pincus, 1936) that whatever its appearance, the epithelium is, in fact, abnormal, in so far as X-rays have suppressed its capacity for oogenesis. The evidence also lends itself to a third interpretation. Everett (1943), whose observations suggest that the 'somatic epithelial tissue' of the germinal epithelium is distinct from its germ-cell producing elements, considers that such X-ray experiments merely show that the 'somatic epithelial tissue'—the surface germinal epithelium which never produces ova—is not damaged.

The fact that grafted ovarian tissue may contain both follicles in active development and luteal tissue long after it is implanted may also be taken as an indication that the germinal epithelium is not essential for oogenesis. This line of evidence, however, hinges on two sets of considerations—the clear demonstration that germinal epithelium is no longer present in the graft, and the speed at which it disappears after grafting.

Some writers, e.g. Pincus (1936), hold that the germinal epithelium of grafts does not normally disappear; while others, e.g., Herlitzka (1900) and Marshall & Jolly (1907, 1908), state that it usually disappears entirely. The latter authors have reported normal ovarian tissue, presumably without germinal epithelium, in an autograft in a rat after 14½ months. The survival of the germinal epithelium, however, clearly varies with circumstances. Pettinari (1928) suggests that the cells are destroyed when they become adherent to any other tissue. Everett's (1943) experiments seem to confirm this view. When he transplanted ovaries within their bursa on to the kidneys of mice they frequently took without fusion, and oogenesis continued. When they were transplanted without the bursa, fusion occurred, the germinal epithelium disappeared, and oogenesis ceased. In the experiments described in the present paper, and also in those of Breward & Zuckerman (1949), the germinal epithelium was undoubtedly replaced by other cellular elements—especially where it was adherent to another tissue. On the other hand, in these experiments oogenesis, as manifested by the presence of young follicles, had not stopped.

The speed with which the germinal epithelium of an ovarian graft changes has been remarked upon by several authors. According to Athias (1920) and Pettinari (1928) swelling and hyperplasia occur within 2 or 3 days, and are associated with many signs of degeneration. Athias found cords of germinal epithelium growing into the cortical zone of the graft, and he suggests that ova are formed from these downgrowths. Pettinari, however, who has observed the same change, regards it as a reaction to irritation and not an oogenetic phenomenon. Tamura (1927) has addresssed himself closely to the question of the survival of the germinal epithelium, and has made an extensive series of ovarian grafts onto the kidneys of male mice, the tissue being placed just within the kidney capsule. His observations were limited to the first 35 days after grafting. He found that in general the follicles existing in a graft at operation disappear, and that they are replaced by the germinal epithelium which remains functional and normal in appearance. He makes the interesting observation, however, that where adhesion occurs, the germinal epithelium disappears and he therefore concludes that 'if the germinal epithelium is unimpaired it proliferates'. Hannan (1929), however, reports the very rapid disappearance of the germinal epithelium of ovarian homografts in the deep fascia on the backs of rabbits. Butcher (1932) also refers to the disappearance of germinal epithelium in some ovarian autografts in the kidney fat of rats. In others he describes the persistence of the epithelium. His work, like Tamura's, also suggests that the germinal epithelium is in general more resistant to impaired vascular conditions than are the existing ova and follicles, which it replaces by proliferation.

It is obvious that the fate of the germinal epithelium in ovarian grafts is very variable. It is equally clear that it is almost impossible to be sure that an epithelium that has apparently been replaced by connective tissue does not persist functionally either within that tissue or as ingrowths into the substance of the ovarian tissue. Desaive (1942) has claimed that 'pre-ova' can be recognized within the ovarian stroma. Derivatives of the germinal epithelium which are not definitive ova may very well be present as 'pre-ova' within the substance of an ovarian graft.

It would also seem that more has been made than the evidence warrants of the 'disappearance of germ cells in ageing grafted ovarian tissue. The finding has

usually been taken to support the view that the germinal epithelium is essential to oogenesis. In the observations reported in the present paper the number of ova per grafted ovary was not one-hundredth of the normal. Corresponding observations have been made by other workers. Nevertheless, it would be quite unjustifiable to assume, even though it may be the correct explanation, that this is so because no germinal epithelium was present, or that the same picture would have been seen if the tissues had been studied a year later than they were. Before reaching so final a conclusion, it would be necessary to carry out a series of quantitative experiments in which different-sized ovarian fragments were implanted into different sites, and examined at considerably longer intervals after grafting than has been the rule. Only if it were shown that there is in general a time-correlation in the disappearance of ova from grafts whose germinal epithelium had been lost could it be argued that in these circumstances ovarian stroma is unable to replace germinal elements.

A new factor has recently been introduced into the problem by the emphasis which Dornfeld and his colleagues lay on the differential proliferative activity of the area of germinal epithelium to which the suspensory ligament of the ovary is attached (Slater & Dornfeld, 1945; Vincent & Dornfeld, 1948). According to these workers, this represents almost the only site at which the germinal epithelium of the rat shows proliferative activity—at any rate up to the sixtieth day of life. Their view is that the associated parts of the oviduct and fimbria, through the ribonucleic acid they produce, exercise a stimulating and evocative effect on the adjacent area of the germinal epithelium, which at the hilum forms a solid block of cells in the substance of the ovary. Several points need to be settled before this hypothesis can be related to our knowledge of the behaviour of ovarian grafts. The existence of an area of hyper-activity in the germinal epithelium of mature animals of several species has first to be established. Its dependence on an induction stimulus from the oviduct also needs to be established. Even if oogenesis in grafts lacking a germinal epithelium can be related to an ingrowth of germinal epithelium similar to the one which Dornfeld and his colleagues have shown in the rat, it is clear, in experiments of the kind reported in the present paper, that it cannot be attributed to the local diffusion of a chemical stimulus from the oviduct.

SUMMARY

1. The ovaries of two rhesus monkeys (*Macacus mulatta*) were transplanted as whole autografts into the eyeballs. In the first experiment (Mm. 77) they became established in the vitreous of one side, and in the sclera of the other. In the second (Mm. 88) they became fixed in the vitreous on both sides. The first animal lived 10 months and the second 11 months after the transplantation.

2. Cyclical uterine bleeding occurred in both animals after the transplantation.

3. In the first animal one ovary contained a number of small follicles undergoing atresia, and a few cystic follicles. The other ovary, which had embedded itself in the sclera, contained many healthy follicles and also three distinct and two ill-defined corpora lutea.

4. In the second experiment, in which both ovaries had become fixed in the posterior chamber, neither contained any healthy follicles or corpora lutea. Soon after their appearance ova underwent atresia.

5. Germinal epithelium was present in only one of the four ovaries. None was present in the ovary which had embedded itself in the sclera, and which was the most active of the four.

6. These observations are discussed in relation to general views about the part played by the germinal epithelium in oogenesis.

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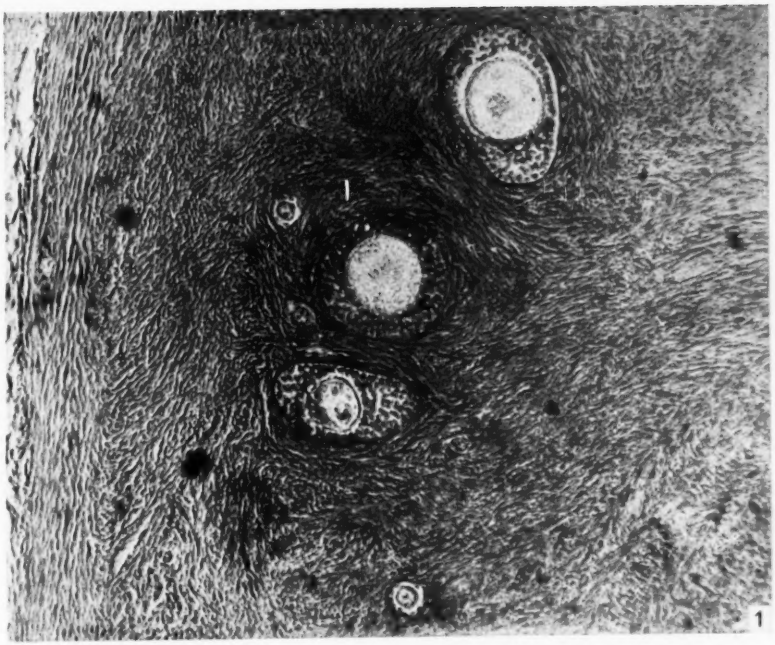
EXPLANATION OF PLATE

Fig. 1. Young healthy follicles in peripheral part of right ovary of Mm. 77, showing the absence of germinal epithelium. ×120.

Fig. 2. Corpus luteum in right ovary of Mm. 77. ×50.



MANDL AND ZUCKERMAN—OVARIAN AUTOGRAFTS IN MONKEYS

STUDIES ON HAIR GROWTH IN THE RAT

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In seeking to answer such questions as, what makes a hair start growing or cease growing, it is necessary to increase our knowledge of the structure of hair follicles and their immediate environment, and particularly their relation to the vascular system. The first part of this paper is concerned with these structural aspects, while the later sections deal with the experimental investigation of the transmission of growth from regions of activity into regions where the hair follicles are quiescent.

The mode of hair replacement varies in different animals and has a constancy in relation to species. Three principal types of follicle activity determine the various patterns:

(1) Continuously active as in wool and human head hair.

(2) Periods of activity and inactivity with neighbouring follicles in different phases.

(3) Periodic activity with neighbouring follicles in the same phase.

The third type is characteristic of the adult rat where active growth is confined to a relatively narrow zone, commencing ventrally and spreading dorsally over the trunk so that at a given time the major portion of the skin is non-active. In the head region and around the limbs the pattern is more complex, but the same essential feature is invariable, viz. the periodic succession of growth waves and intervals of quiescence. Some details of the growth pattern for the rat have been given by Dry (1928), Butcher (1934) and Haddow, Elson, Roe, Rudall & Timmis (1945). The position of the wave may be demonstrated very easily in the living animal by clipping the hair over one side and then observing in the following few days the band along which rapid hair growth appears. Another method which is very useful in the case of white rats is to dye the coat with proflavine and then observe the position of the growth wave by the appearance of non-dyed new hair which stands out in sharp contrast to the dyed hair of the quiescent areas (Pl. 1, fig. 1; Pl. 4, fig. 10). In the present work the method of clipping was used in most cases.

The regularity of the growth pattern is quite characteristic in post-pubertal animals of both sexes, but there is some disturbance of the pattern in old age, during lactation, and in states of nutritional deficiency and disease. Our main observations have, therefore, been made in non-pregnant, pre-senile animals so as to avoid complicating factors. Stock albino rats were used throughout, except that in the denervation experiments hooded and black rats were used as well as albinos.

HISTOLOGY

Microscopic examination of skin sections in the region of a laterally placed wave shows that structural changes are present in a sharply defined zone that corresponds in position with the observed wave of hair growth in the living animal (Pl. 2, figs. 2 and 3). The inactive hair follicles lie quite high in the dermis and do not extend down

as far as the panniculus carnosus. In the zone of active growth, on the other hand, the roots of the hairs extend down to the panniculus and are even sometimes deflected and grow along for a distance parallel to the panniculus giving the hair follicle a characteristic 'hockey-stick' appearance, previously observed by Butcher (1934). Within an active zone all hairs are involved in the process of growth; large and small follicles extend to nearly the same depth.

Coincident with hair growth there is a characteristic increase in the amount of hypodermic fat which gives an added thickness to the whole skin (Pl. 2, fig. 2), and in sections across the wave a clearly defined band or ridge is evident. This is due to hypodermic increase and not to any appreciable change in the thickness of either the dermis itself or the epithelium (cf. figs. 4 and 5, Pl. 2). Thickening of the skin during active hair growth was observed by Butcher (1934) in a comparison of a series of animals of increasing ages, mainly prepubertal. The same author (1937) has described the variations in fat content of the hypodermis in autografts and homografts of the skin of white rats and he noted the very general—though not invariable—association of increased fat deposition with hair growth. The illustrations in our Pl. 2 show clearly the variation in thickness of the hypodermis across a wave in individual adult rats, and they depict standard differences between fully active and fully inactive growth zones. In order to study details at the advancing edge of the wave, samples of skin must be excised during one of the new pulses of growth. These cannot be recognized until they are so advanced that the new hair has already reached above the skin surface and these features are not included in the present study.

The other concomitant of the growth wave is the increased vascularity which again is confined sharply to the band of skin in which hair growth is active (see Haddow & Rudall, 1945). In our present studies, indian ink injection of the whole animal through the heart or aorta was used: whole mounts of cleared portions of the skin and also sections of the appropriate regions were examined. The capillary bed within the wave of growth was densely injected in contrast to the condition in adjacent quiescent skin. The main concentration of capillaries was around the lower portion of the external root sheath and about the bulb, while the papilla of the hair follicle usually failed to show any injection. The presence of vessels within the papilla is, however, not in doubt (Pl. 3, fig. 6). The richness of the basket-like network of capillaries around the lower part of the follicle is clearly shown in Pl. 3, fig. 7, C and E. A difficulty inherent in the procedure of injection is that equivalent areas are not always equally well injected. Studies were confined to regions which appeared to be more completely injected.

A picture of the variation in the vascular supply to the follicles across the wave of growth is given in Pl. 3, fig. 7. The sections were made in a dorsoventral plane lying parallel to the hair slope, and suitably oriented follicles were photographed at various positions across the wave-front from the advancing edge A, to the receding edge G. The larger types of follicles illustrated in Pl. 3, fig. 7, with their capillary systems, are of the monotrich and awl classes (see Dry, 1926, 1928), but the capillary systems of follicles of the smaller types are comparatively ill-defined, i.e. even less developed than in D. The group of follicles A is in the resting stage and shows the minimal development of the capillary system. The group consists of a large mono-

trich (or awl) and a number of smaller types. The whole group is supplied by a series of capillaries arising from larger vessels placed more deeply in the dermis. The follicle B is of the monotrich or awl class, and was found at the advancing edge of the wave-band. The hair is being produced, and its pointed tip has just penetrated beyond the skin surface; at the same time the follicle is growing down into the hypodermis and a rich capillary network is developing at its base. The smaller follicles of the group shown in B are delayed in development and have not descended so far into the dermis. (It is occasionally noticed, in observing regrowth of hair in the rat after shearing, that the monotrichs attain a length of at least 1 cm. before the other fibre types appear above the surface.)

The two follicles C and D were growing side by side, C being at the advancing edge of the growth wave and D next to it inside the growth zone. The fibre from C is a typical monotrich with a thick cortex and a thinner medulla. The fibre from D is a typical awl. The follicle E is situated near the receding edge of the band of growth and its fibre, which is still growing vigorously, shows a thick cortex and is thus of the monotrich type. The capillary supply to the lower half of E is richly developed as it is in other monotrich follicles, e.g. at C.

At the receding edge of the wave the follicles are shortening, and the levels of the bulbs are retracted towards the surface. In the follicle F the fibre has lost its medullation and the hair 'club' is in an early stage of formation. The follicle F may appear to be truncated basally, but this is not really so; the section is thick and the capillaries of both the lower and the upper surfaces of the follicle cylinder can be focused to the base. In polarized light the beginning of marked anisotropy in the cortex was found and this level was at the normal distance from the base of the whole follicle. The capillary network of this much shortened follicle is reduced, and appears to be contracted in a longitudinal direction. The capillary network originally deeply situated in the hypodermis, as at C and E, has in F been carried towards the epidermis by the processes involved in follicle shortening.

In G we see the beginning of the resting period, with the hair 'club' well formed. The capillary network, which has been carried up from the hypodermic level, appears contracted and is receding towards a minimal system as seen in A.

It thus appears that the capillary system to the follicle wall degenerates during the retraction of the follicle towards the surface, especially at the end of the retraction process, and that all follicles of a group which have been resting for a full term, as at A, still possess an active, though reduced, capillary system.

The type of capillary supply to the periodically growing hair follicles of the rat was compared with that to the continuously growing wool follicles of the sheep. Suitable preparations were obtained by injecting an advanced sheep foetus through the umbilical vein with soluble Prussian blue, followed by fixation in formalin. Such injections, though not complete, generally showed the capillaries of the papillae to be well injected. The base of the follicle is not richly supplied, and the maximum concentration of capillaries lies just above the level of the papilla around the thickest part of the external root sheath (Pl. 3, fig. 8). There is thus an obvious difference between the capillary supply to the large hair follicles of the rat and to the wool follicles of the sheep in respect of both the kind of distribution and the quantity of the supply.

There was no evidence that the vessels supplying the capillaries associated with the hairs were isolated from other cutaneous systems. There is, probably, continuity between vessels of the superficial and the deep layers, including those in the panniculus. When mammary gland tissue is present in sections of injected rat material it is seen to be richly supplied with capillaries (Pl. 4, fig. 9). During lactation in the rat there is some interference with the normal pattern of hair growth, while reduction of hair growth during lactation has been recorded in experiments with guinea-pigs and sheep (Strangeways, 1933; Bosman, 1935). If the activity of the mammary gland in any way diverts the blood from other parts of the cutaneous field, an alteration in hair growth would not be surprising in view of the evident association between hair growth and vascularity. Further, it has been reported (Mottram, 1945) that in mice, which also present a periodic hair-growth pattern, epidermal warts and the epidermis show reduced growth during the passage of the wave, and resume active growth after the wave has moved on; here again one can see a possible connexion between the observed phenomenon and the condition of the vascular bed.

EXPERIMENTAL OBSERVATIONS

Various experiments were devised in order to examine the factors which influence the waves of hair growth.

A. *Effect of denervation*

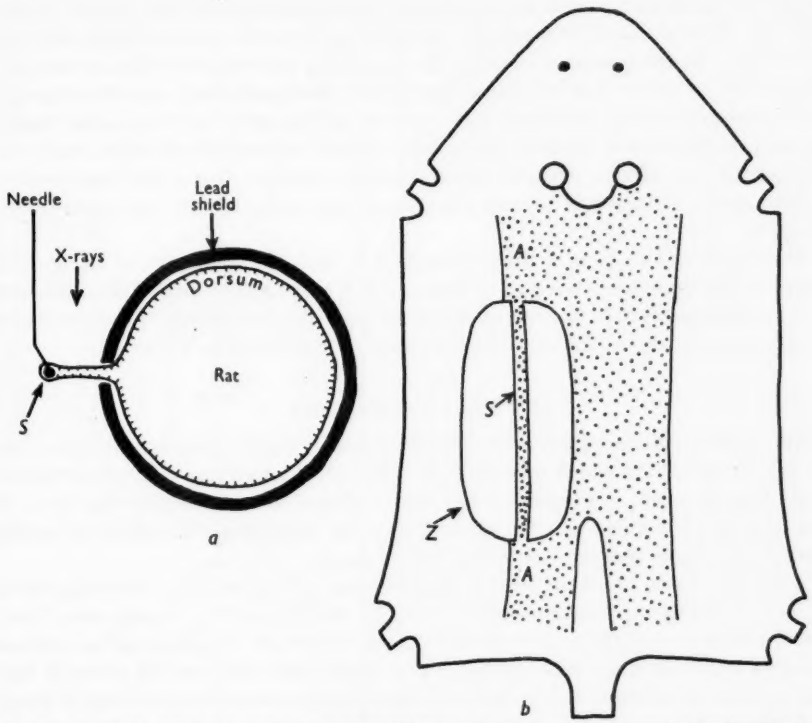
A longitudinal incision was made on one side of the body parallel to the mid-dorsal line from the level of the shoulder to the hip, and the skin retracted sufficiently to allow exposure of the segmental nerves. These were picked up with a fine curved needle and severed so that the major portion of the body wall from shoulder to hip was denervated, the only nerves remaining intact being those derived from the posterior primary divisions and not concerned with skin innervation except in the dorsal skin medial to the incision. The nerves to the panniculus carnosus were severed at the rostral end of the incision: they were easily located deep to the panniculus as a leash of fine branches and, although there is no evidence that they are cutaneous in the usual sense, their severance removed any possibility of their playing a part in the subsequent hair-growth pattern. The incision was closed by sutures, and the wound allowed to heal prior to making any observation of the wave. These animals were observed for periods of 3-5 months following the operation and no alteration in the pattern of growth was detected. Occasionally there was a suggestion of rather less vigorous growth of hair on the operated side, but in all cases the growth wave retained its normal pattern of ventrodorsal progression and the symmetry of the process on the two sides was retained.

B. *Effect of exposure to X-rays*

An anaesthetized animal was enclosed in a lead-protecting shield, along the side of which a narrow slot had been cut. A longitudinal fold of skin was withdrawn through the slot and exposed to a dose of X-rays (3000 r.).* A steel needle, inserted subcutaneously, supported the crest of the fold and this cast a shadow on the skin

* Dose, 3000 r.; dose rate, 315 r./min.; radiation, 80 kV.; half-value layer, 1·3 mm. Al.

(Text-fig. 1*a*). The result was an epilated zone of skin approximately 4 cm. long and 2 cm. broad, with a narrow non-epilated strip *S* running along the middle of the zone (Text-fig. 1*b*). During subsequent observations over a period of 8 months, a very scanty growth of hair was observed on the irradiated area, and it was assumed that the activity of most of the follicles had been stopped. Observation of growth on the two sides of the animal showed that the wave was not altered by the presence of the large epilated area. The wave passed towards the dorsal line in front of, behind and



Text-fig. 1. *a*, Method adopted to irradiate an isolated area of skin. Needle prevents irradiation of skin at *S*. *b*, Diagram of rat pelt to show zone of epilation, *Z*, on left side. *A*, active hair growth. *S*, strip of non-irradiated skin running through epilated zone, *Z*.

above the epilated area, symmetrically with the wave on the opposite side. Within the irradiated area the narrow strip of non-irradiated skin, *S*, came into activity simultaneously with *A* at the same level on the main region of the body. A correspondence between growth at *S* and *A* occurred during each of the four cycles of growth observed.

It must be pointed out that the degree of epilation, though extensive, was not complete. Choosing the most suitable dosage creates problems in itself. Observations made during the course of the present experiments suggest that the necessary epilation dosage depends on the phase of the hair growth at the time of irradiation.

C. *Effect of skin excision*

Strips of skin nearly 1 cm. wide (down to the hypodermis but not including the panniculus carnosus) were removed from the left side in two animals. These strips extended forwards from the hip half-way towards the shoulder in one animal and rather farther in the other. The upper and lower edges of the resulting gap were approximated by sutures and the wound allowed to heal. In the first cycle of hair growth, which followed quickly after the operation, growth proceeded to the scar on the operated side, paused a few days and then proceeded beyond the scar to the mid-line. This revealed little except that the scar line was a region of growth discontinuity. In the second cycle after the operation, growth proceeded to the mid-line in front of the level of the scar on both sides. More posteriorly, growth extended to the mid-line on the non-operated side, but on the operated side it extended during the same period only to the level of the scar. Growth eventually proceeded above the level of the scar after a pause of approximately 2 weeks. One animal survived to a third cycle, and growth beyond the scar on the operated side was again much delayed.

The significant feature of this experiment is that the symmetry of the growth waves on the two sides is affected by removal of a longitudinal strip of skin. Growth is delayed dorsalward of the site of skin removal, either in consequence of removing the skin, or because of the scar which inevitably forms at that level.

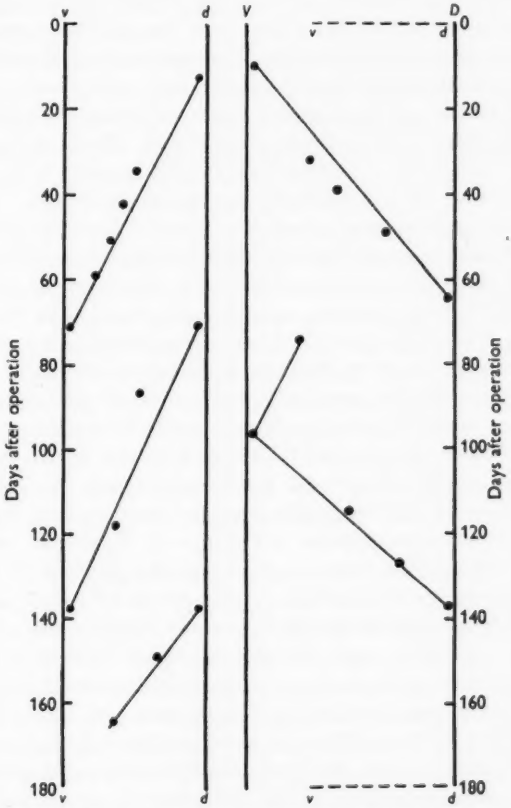
D. *Effect of skin rotation*

The purpose of the experiments described under this heading was to place side by side areas of skin which were in quite different phases of growth. Observations could then be made as to whether the phases altered so as to become the same, or whether they remained as different as before the operation. A number of young post-pubertal rats was treated in the following way.

A square of skin (3×3 cm.) was outlined on one side by incisions extending down to, but not including, the panniculus carnosus; the edges of this square were freed, but the main area of the square was not detached from its subcutaneous connexions so as to leave an intact blood supply. The square was then rotated through 180° and sutured in position. This meant that the original ventral margin became dorsal and the caudal margin became rostral. Healing occurred readily, and in 10 days the site of operation was clearly defined by the reversed hair slope on the square of rotated skin (see Trotter & Dawson, 1931, for similar result in guinea-pig). Observations over a period of several months were then made on the growth waves of the hair. It was found that within the rotated square the growth wave was reversed and passed from the dorsal to the ventral margin. To demonstrate this effect an animal was subjected to the above operation and after healing of the wound the whole fur was dyed with proflavine. Two weeks later, it was noted that the fur was growing white on the upper third of the rotated area, while on the body surface outside this area the new white hair was coming in along a ventrolateral zone. Ten days later the animal was photographed in colour, and a black and white reproduction of this is given in Pl. 4, fig. 10. Within the rotated area growth has proceeded downwards from the upper edge, while on the rest of the body, growth of hair has proceeded

upwards as far as the mid-lateral line. As usual, at the lines of incision there is some distortion of hair slope; this may be noted along the caudal incision in Pl. 4, fig. 10.

The chart shown in Text-fig. 2 was constructed from an extended series of observations on another animal and shows the progress of the growth wave from dorsal to ventral regions within the rotated area of skin *d*- - - - -*v*. All the experimental



Text-fig. 2. Chart to illustrate progress of successive hair-growth waves on rotated skin, *d–v*, in comparison with waves on main area of body, *V–D*. The position of the rotated area on the body surface with reference to the mid-dorsal line (*D*) and mid-ventral line (*V*) is indicated by the interrupted line *v*- - - - - -*d*.

animals tended to conform to this plan. In none of the animals did the movement of growth on the rotated skin proceed in other than a dorsoventral direction; nor was there any suggestion of interference between the ventrodorsal growth on the main areas and the oppositely directed growth on the rotated skin. The general conclusion is that the wave of growth on the rotated skin proceeds independently of that on the main areas, but tends to travel more slowly. In some cases this retardation was very marked.

DISCUSSION

The results of the experiments involving section of nerves to the body wall suggest that the pattern of hair-growth waves in the rat is independent of the innervation of the skin in the regions concerned. It can be assumed that the cutting of the segmental nerves involved not only somatic fibres, but also the sympathetic post-ganglionic fibres travelling in the nerves to the vessels of the body wall including the skin. The vascular pattern associated with the persisting growth wave could not therefore be explained on the grounds of any nervously controlled vasodilatation.

The increased vascularity within the growth wave is largely due to new capillary networks in the dermis and hypodermis. The most extensive capillary network is found around the lower half or third of the follicle. This external supply to the follicle wall is of far greater extent than the internal supply to the follicle papilla, and it is possible that the external root sheath reacts with this capillary system, adding to or removing substances from the blood before this passes through the papillary vessels. In the smaller hair follicles the circulation about the follicle wall is very much less extensive than that shown at C and E (Pl. 3, fig. 7). The follicles which produce the largest overhairs, notably monotrichs, have the most extensive capillary system. These fibre types contain a considerable bulk of keratin, as can be seen in fig. 7 of the paper by Haddow & Rudall (1945), and a most reasonable explanation of the extensive circulation to the follicle wall would be that it is associated with the synthesis of intracellular protein in the hair cuticle and cortex.

In our studies of the follicle circulation we have not been able to observe the capillaries to the papilla except in a few isolated cases. According to Johnson, Butcher & Bevelander (1945) the papilla markedly alters its shape during the catagen phase (Dry, 1926), becoming narrow and elongated. There may well be changes in the circulation to the papilla at this time, though the circulation to the follicle wall does not appear to change. It should be pointed out that the distribution of capillary vessels corresponds approximately with that of the supposed phosphatase activity in the papilla and the follicle wall. As the capillaries themselves show abundant phosphatase activity it is not certain to what extent the recorded distribution of the enzyme is due to a blurred distribution of capillaries. On such a view the reduced phosphatase activity of the papilla at the catagen phase may represent the reduction or degeneration of its capillaries. But if we accept the recorded distribution of alkaline phosphatase as being substantially correct and not due to capillary phosphatase, then the following valuable correlations may be made: the development of this phosphatase activity is associated with the development of a rich network of capillaries, and the disappearance of phosphatase is associated with the disappearance of the special capillary system.

In the initiation of growth at the wave-front it can be debated whether the follicle activity or the increased vascularity has priority. The periodic nature of hair growth would seem to be due to a fixed genetic pattern. Having entered the resting stage, no proximity to an active circulation will cause growth to start again until the required resting stage has been completed. Thus a wave-front moves forward and establishes a new front on meeting follicles which have not rested sufficiently long to recommence activity. It has been shown that there are special capillary branches

to follicles which are in the completely quiescent stage (Pl. 3, fig. 7A), and such follicles may be affected via their residual capillary system by the presence of adjacent highly vascular areas of the type illustrated at B–E. In other words, the presence of an increased vascular system at the wave-front may help to limit the resting period in contiguous areas so that these are generally the next regions to show follicle activity.

The experiments which have been described, involving X-ray epilation, skin excision and skin rotation, were performed with the object of studying the progress of the growth wave in an area isolated in some way from its normal contacts with undisturbed skin. Isolation was achieved in one set of experiments by X-ray epilation and in these cases the possible influence of skin incisions was avoided; the excision of strips of skin or the rotation of an area of skin allowed the juxtaposition of regions in different phases of activity, in the one case by removal of the intermediate phases and in the other by the mere rotation.

The particular form of the epilated area resulting from the X-ray irradiation left an isolated strip of non-irradiated skin (Text-fig. 1). During four cycles of hair growth the narrow non-irradiated strip passed into the active phase at the same time as the non-irradiated areas at the same level elsewhere on the body. There was no evidence that growth on the strip *S* (Text-fig. 1) was in any way delayed because of absence of contact at its ventral edge with a full complement of actively growing follicles. By contrast, where a strip of skin was excised along one side of the body there was a definite delay in the ventrodorsal passage of the growth wave. This is the only example we have at present where conditions have been imposed which produce a barrier to the transmission of the wave and we do not yet know whether this barrier is due to scar formation or to the removal of necessary intermediate phases.

From the skin-rotation experiments we have concluded that the direction of the wave is inherent in the skin; the wave of growth proceeds independently within the rotated area as if the latter were isolated from surrounding skin. Just the opposite conclusions were reached by Butcher (1936) who states 'the time of hair growth is dependent on factors resident within the animal and not in the skin'. The principal differences between our approach and Butcher's were as follows: (1) We have dealt exclusively with adult rats, whereas his experiments were performed on prepubertal material. (2) We studied areas of skin of much greater dimensions than he did. (3) The skin in our rotation experiments was never completely detached from the underlying tissues, and so the blood supply remained relatively intact. These points are sufficient to account for the different findings. From the observations of Heringa & Weidinger (1942) it is reasonable to assume that in prepubertal animals the skin is gradually becoming more fibrous and less highly hydrated. It is also reasonable to suppose that the rate of diffusion of activating substances through the skin decreases with increasing age. On this basis we may be able to account for the fact that the hair-growth waves change from the broad prepubertal type described by Butcher (1934) to the narrow type illustrated in Pl. 1, fig. 1. It is also probable that the structure of the scar tissue will be much more densely fibrous in older animals, and this could account for the 'barrier' effect resulting from excision of strips of skin and for the apparent isolation of rotated areas of skin.

The less rapid growth which we observed on the rotated skin might conceivably be attributed to a varying degree of interference with the normal circulation. In Butcher's experiments, which involved a complete severance of all vascular connexions, he found that the follicles retracted towards the epidermis in the donor skin without completing the normal cycle of growth. The normal resting period of the follicle had not been established and thus its inertia to regrowth was not present. It is therefore understandable that the oncoming vascular wave should stimulate the donor skin to active hair growth. Where the normal cycle of follicle activity was maintained we failed to detect any stimulating effect of one area on another when hitherto unrelated edges were brought into contact by skin rotation. Butcher's experiments indicate, however, that such an effect does in fact exist. Our experiments show that this effect, if present, is small compared with the inertia of the resting period or is decreased in adult animals by the scar tissue.

It seems reasonable to conclude that the progress of the hair-growth wave is due to interaction between the resting-stage inertia and the stimulus of neighbouring vascular activity.

SUMMARY

1. The histological features of the skin and the hypodermis in the rat are compared in areas showing active hair growth and in quiescent areas.

2. The density of the capillary bed about the hair follicles varies according to the stage of the hair-growth cycle. The distribution of capillaries is similar to that of alkaline phosphatase activity.

3. Principal differences between the circulation to hair follicles in the rat and the sheep are described.

4. Denervation of an area of skin has no effect on the passage of the wave of hair growth over it.

5. Various experiments, involving skin excision, skin rotation and X-ray epilation, were performed in order to study the processes involved in the transmission of hair-growth waves from venter to dorsum and we have concluded that this is due to interaction between resting-stage inertia and the stimulus of neighbouring vascular activity.

We are indebted to Dr F. W. Spiers, Lecturer in Medical Physics in the University of Leeds, for his advice concerning the irradiation of the skin and for providing the facilities for this aspect of the investigation. To Mr W. Hutchinson of the Anatomy Department we are grateful for his assistance at operations and for the preparation of the histological material.

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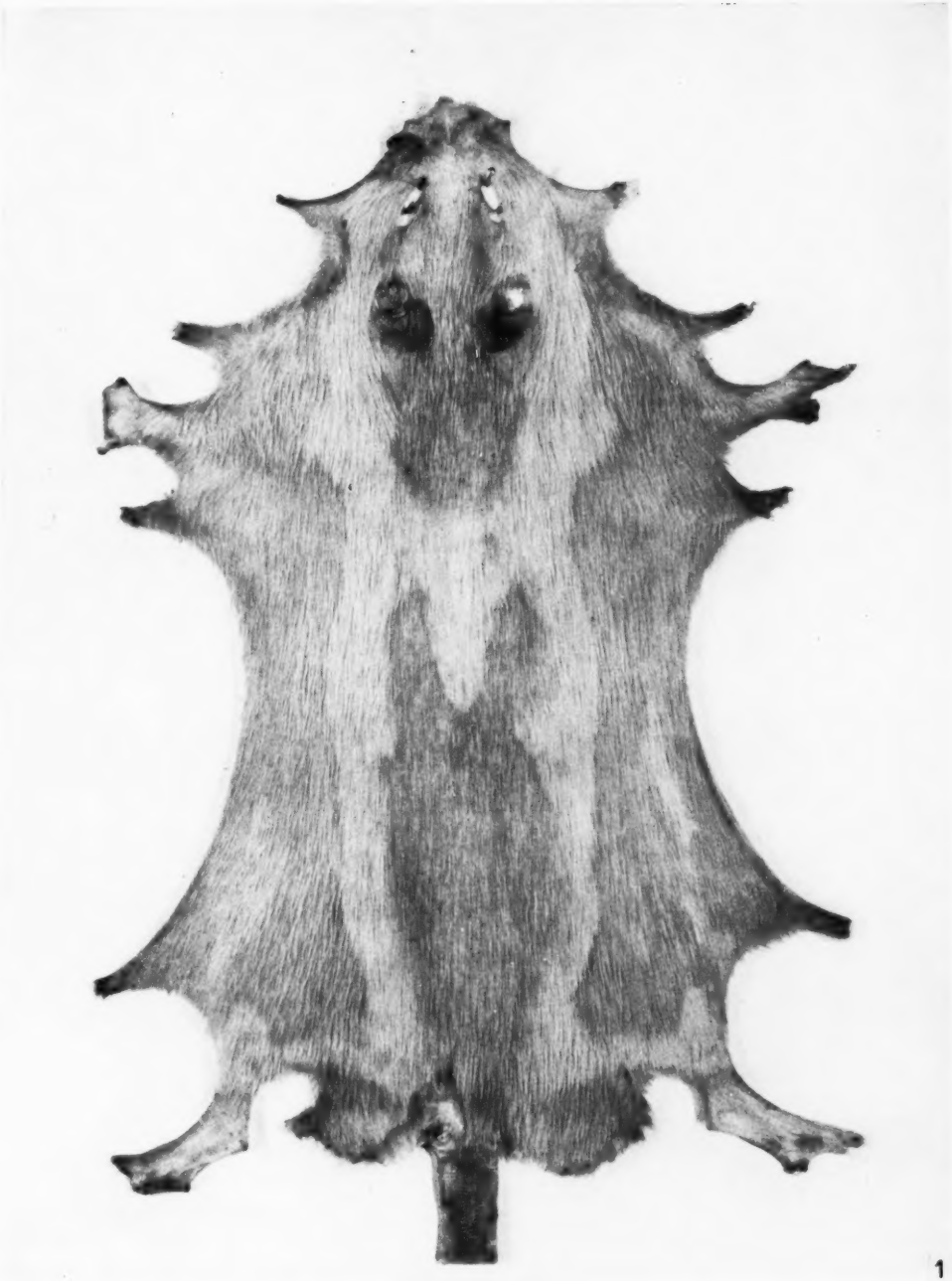

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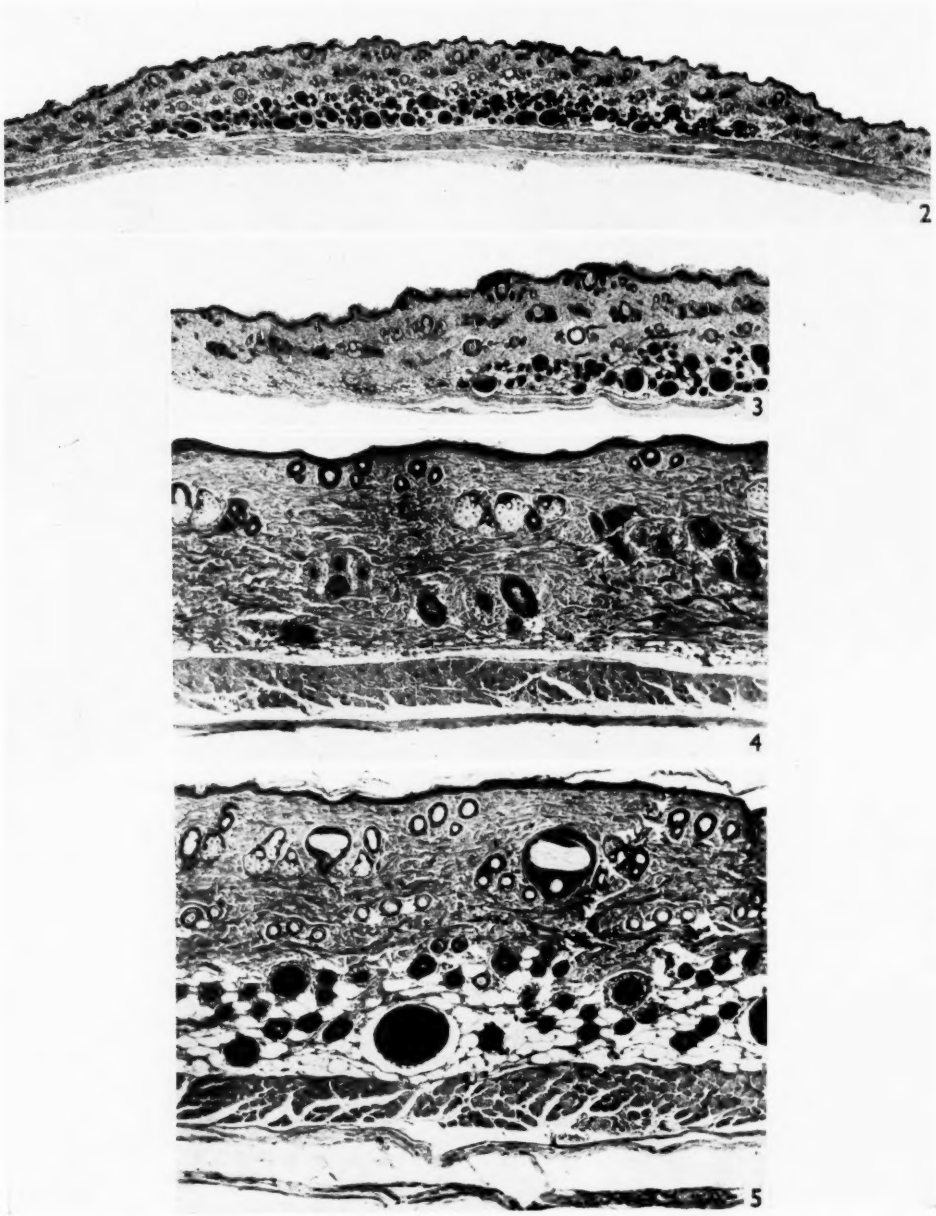
DURWARD AND RUDALL—STUDIES ON HAIR GROWTH IN THE RAT



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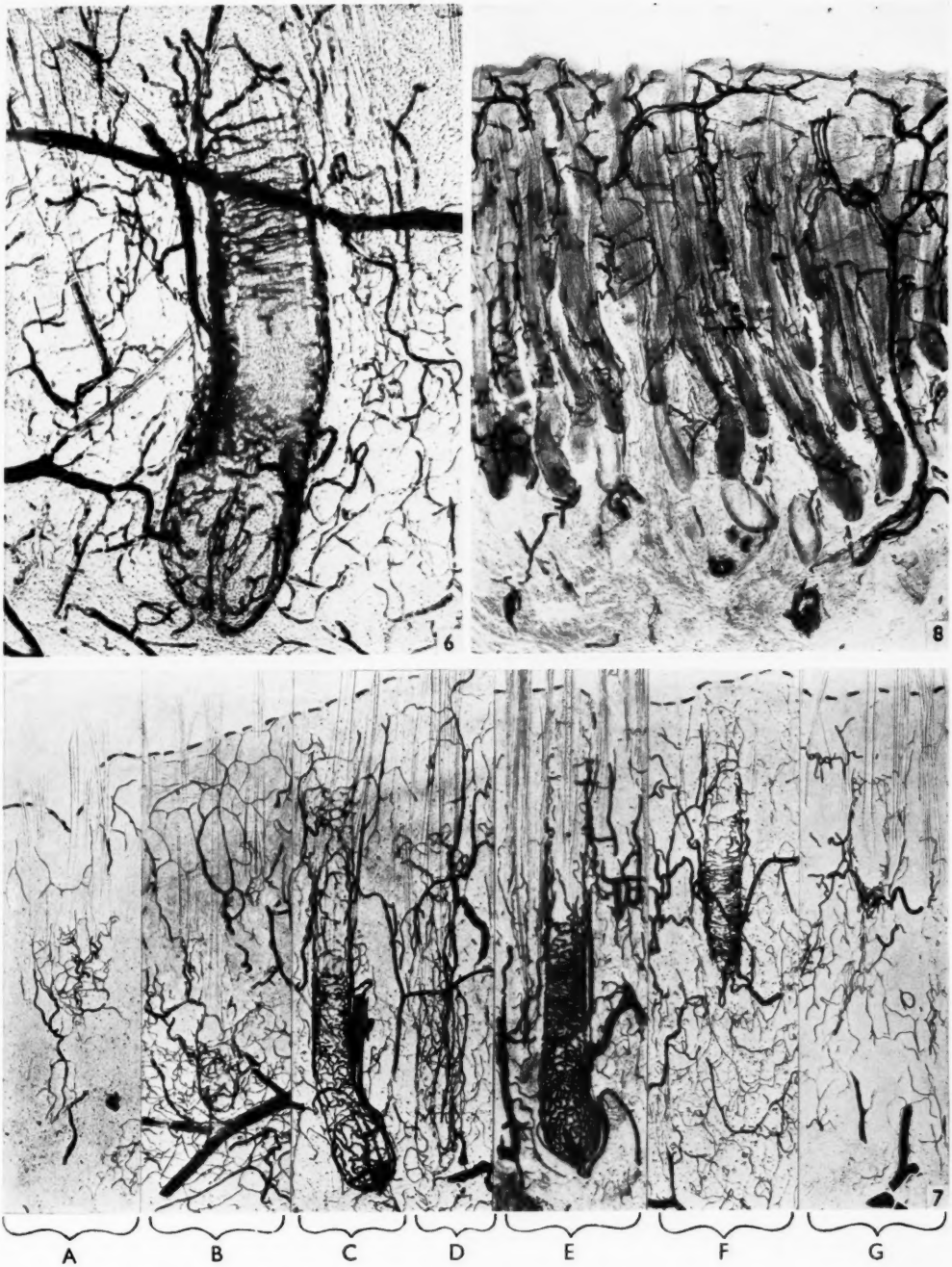

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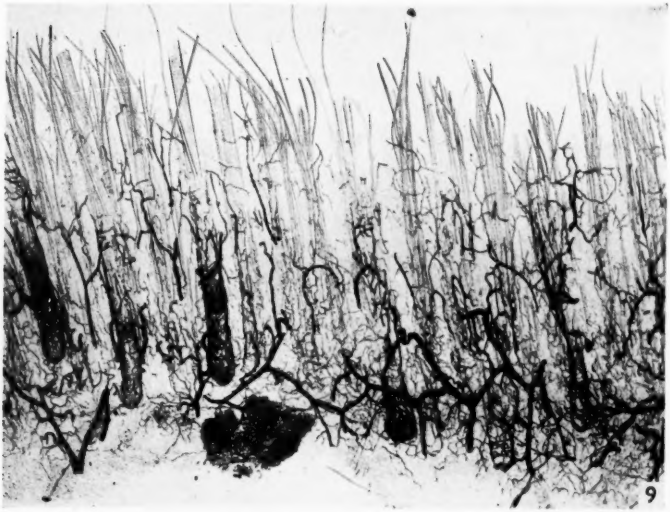

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Pelt of white rat, the coat of which had been dyed with proflavine. Animal killed 27 days later. Light colour shows new growth and darker colour the quiescent areas.

PLATE 2

Fig. 2. Vertical section across growth wave showing the zone of active hair growth centrally and the quiescent zones on either side. Owing to the obliquity of the hair the follicles are cut in the main transversely. Stain: Ehrlich's haematoxylin and eosin. ×20.

Fig. 3. The junction between active (right) and quiescent (left) zones. The variation in fat content of the hypodermis in the two zones is evident. Stain: Ehrlich's haematoxylin and eosin. ×23.

Fig. 4. Vertical section of skin in quiescent area. The fatty hypodermic layer between the dermis and the panniculus carnosus is seen to be thin. This is from the same section as fig. 5. Masson's triple stain. ×73.

Fig. 5. Vertical section through active zone. The thickness of the hypodermic layer contrasts with the condition seen in fig. 4 taken from the same section. Masson's triple stain. ×73.

PLATE 3

Fig. 6. Follicle from injected skin to show the capillary network within the papilla. Indian ink injection. ×80.

Fig. 7. A series of injected hair follicles through a wave of growth from advancing to receding margin. Explanation in text. Level of epidermis indicated by dotted line. Indian ink injection. ×35.

Fig. 8. Vertical section of skin from injected sheep foetus. Prussian blue injection. ×48.

PLATE 4

Fig. 9. Section of skin, parallel with hair slope, from a pregnant rat showing a portion of heavily injected mammary gland. Indian ink injection. ×16.

Fig. 10. Skin rotation on a white rat demonstrating dorsoventral progression of growth on graft in contrast with ventrodorsal growth on main areas. For details see text. Light areas represent new growing hair and dark areas quiescent hair.

INTERNODE LENGTHS IN THE NERVES OF FISHES

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Department of Anatomy, University College, London

INTRODUCTION

Studies on mammals and amphibians have begun to reveal the principles that govern the spacing of the nodes of Ranvier. These gaps in the myelin are normally placed farther apart in larger than in smaller fibres (Ranvier, 1875, and others; see discussion later), but after regeneration in the adult this relationship no longer obtains, all internodes are then short (Young, 1945*b*; Sanders & Whitteridge, 1946; Hiscoe, 1947; Vizoso & Young, 1948). This suggests a connexion between growth and internode length, which is confirmed by the finding that the line relating internode length to diameter becomes progressively steeper during development (Young, 1945*b*; Hiscoe, 1947; Vizoso & Young, 1948). Further, in the legs of amphibians the internodes are longest where the amount of growth is greatest (Takahashi, 1908; Hatai, 1910).

The few studies made on fishes (Ranvier, 1872; Key & Retzius, 1876) show that these animals possess internodes several times longer than any reported from mammals or amphibians. We have therefore studied the internode length in nerves of fishes of various lengths, and also in nerves taken from parts of the body likely to have differing growth rates. In particular we have been interested in long-bodied fishes, such as the eels, where the nerves increase several times in length during the period after medullation.

METHODS

The nerves, with the exception of those from *Torpedo*, were all obtained at Plymouth during the summer of 1947 from the elasmobranchs *Raia clavata*, *Scyliorhinus canicula* and *Torpedo ocellata*, and the teleostean *Conger conger*. The nerves used were mostly the lateral line and branchial branches of the vagus. After dissection, the stretch of nerve to be taken was measured while still in position, then cut with scissors and attached to a piece of slotted cardboard at its original length. It was fixed in 10 parts of 40 % formaldehyde diluted with 45 parts of sea water and 45 parts of distilled water. The salts in such a solution will be approximately isotonic with those in the blood (Young, 1933). The nerves were stored in this medium until required some months later. They were then washed, and stained with a 1 % solution of osmium tetroxide for 24 hr. After further washing in distilled water they were passed to a mixture of glycerin and water in the proportion of 2 to 1 for 3 days to facilitate teasing.

Single fibres were obtained by dissociation in pure glycerin under a dissecting microscope with fine mounted needles, care being taken to minimize damage or stretching. The internodes may be up to 8 mm. long, so isolation for considerable distances was required. With careful dissection lengths of 4 cm. or more could be obtained.

After isolation, single fibres were transferred to a slide on which drops of creosote had been placed. After several fibres had been collected in this manner the creosote was drained off, the slide blotted and the fibres mounted in Canada balsam. Displacement was avoided by using balsam of a low viscosity. The slides were dried in an incubator at 37° C. for 1–2 weeks before measurements were carried out.

Measurements were made using an ocular micrometer (Zeiss Compens-Okular, × 12). Those of internodal length were carried out using a $\frac{2}{3}$ in. objective, the draw-tube being adjusted to give a magnification of × 100. Readings of the diameter were taken under a $\frac{1}{12}$ in. oil-immersion objective at a magnification of × 1000. Ten readings were made along each internode, at approximately equal distances apart. By this method it was possible to measure internodal distance to the nearest 10 μ and diameter to the nearest 1 μ.

ERRORS DUE TO FIXATION AND DEHYDRATION

As the stretches of nerve were attached to cards by both ends throughout fixation and were only removed immediately before teasing, no change in length took place during fixation. With regard to diameter Rexed (1944) found a very slight swelling, not greater than 1·25 %, of the small fibres, after treatment with 4 % formaldehyde; he also reported no change after prolonged storage (up to 4 months) in formalin. Rexed and others have found no diameter change after treatment with osmium tetroxide, although some have reported varying amounts of shrinkage.

To assess the error introduced by dehydration, we measured fibres before dehydration, when in the glycerin and water mixture, again after having been in pure glycerin for 2 hr. and finally after mounting in Canada balsam. Shrinkage occurred mainly in the latter stage and the total amounted to $2{\cdot}3 \pm 0{\cdot}73$ %, affecting length and diameter to the same degree.

TREATMENT OF DATA

For each nerve, the lengths of individual internodes were plotted graphically against the mean of the ten corresponding diameter readings. Further treatment is described in the relevant sections. Where regression lines have been fitted, the method of least squares has been employed.

RESULTS

(1) *The forms of the nodes*

Ranvier (1872) drew attention to the fact that in the ray the myelin tapers towards the node. This is true not only in *Raia* (Pl. 1, figs. 1–3), but also in *Scyliorhinus* (Pl. 1, fig. 4) and *Torpedo* (Pl. 1, fig. 5). The nodes in the conger eel, however, closely resemble those of mammals in the smoothly rounded contours of the myelin, the ends of the two segments appearing as if closely pressed together (Pl. 1, fig. 6).

Ranvier also reported that the nodal axon in the rays is stained black with osmic acid. In our own preparations there appears to be in most instances a definite interruption of the myelin (Pl. 1, figs 1–4), the demarcation of the limits of the sheath being at times very striking (Pl. 1, fig. 4). At times the region of the node is osmiophilic to some degree, this being most frequently noticed in *Torpedo* (Pl. 1, fig. 5). It is probable that the axon surface contains a proportion of lipoids similar to

that in non-medullated nerves (Schmitt & Bear, 1939) even where not covered by a thick layer of 'myelin'.

The nodes were usually easily recognized and distinguished from accidental breaks in the fibres or from other artefacts.

(2) *Length of internodes*

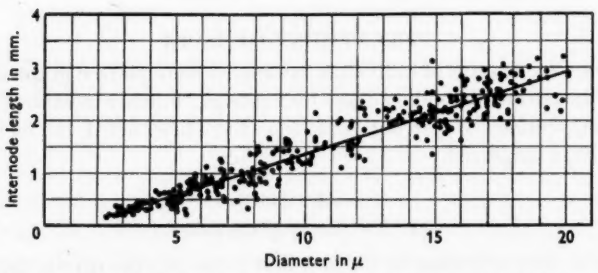
Internodal lengths were found to be even greater than Ranvier supposed. In the lateral line nerves of a ray 83 cm. in length and a conger eel 124 cm. long the greatest mean internodal lengths found were 7·02 and 7·52 mm. on fibres of 22·0 and 26·5 μ diameter respectively; in a dogfish 65 cm. long the corresponding value was 4·90 mm. on fibres of 19·4 μ. These may be contrasted with the maximum of about 1·5 mm. found in the rabbit on the largest fibres (20 μ diameter). Kubo & Yuge (1938) reported lengths up to 3·7 mm. in the toad.

The smallest fibres in our series are about 2·5 μ in diameter and have internodal lengths of about 0·20 mm., which is similar to that found in the small fibres of amphibians and mammals (see p. 346).

(3) *Correlations between internodal length and diameter*

Previous work on this question in fishes has been carried out by Ranvier (1872) and Key & Retzius (1876). The former stated that there exists in the rays a direct relationship, such that the larger fibres have a longer and the smaller a shorter internodal distance. A limited number of observations are given by Key & Retzius for the pike, but their data show little variation of length with diameter.

We have found that there is a positive correlation between internodal length and diameter, and, as in other vertebrates, the relationship is approximately linear.



Text-fig. 1. Internodes from lateral line nerve of a small specimen of *Raia* (40 cm. in length).

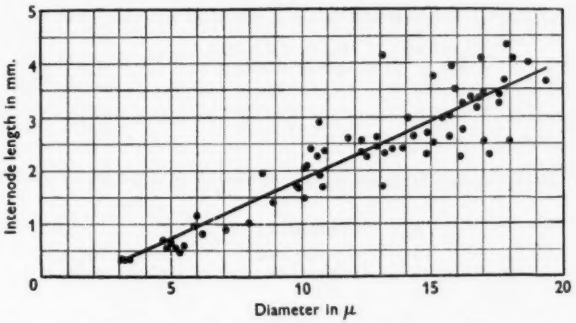
Text-fig. 1 is of fibres from the lateral line branch of the vagus of a specimen of *Raia*, 40 cm. long. A regression line has been fitted, and if

$$y = a + bx,$$

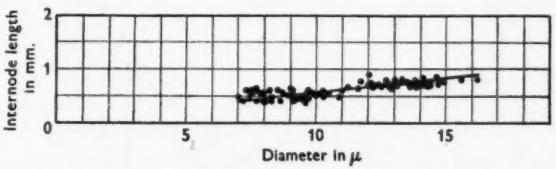
where y represents the internode length in mm., x the average diameter per internode in μ, a the y-axis intercept and b the slope coefficient, a has the value of $-0{\cdot}14$ mm. and b, $0{\cdot}15 \pm 0{\cdot}00001$.

Text-fig. 2 gives the measurements from the lateral line nerve of a specimen of *Conger*, 63 cm. in length, the constants being $a = 0{\cdot}38$ and $b = 0{\cdot}22 \pm 0{\cdot}0001$.

Text-fig. 3 is of the nerve to the electric organ of *Torpedo*. This shows a situation different from that of the lateral line nerves in that there is only a very slight increase of internode length with diameter; $b=0{\cdot}053$. Moreover, a further peculiar feature is that there are no fibres smaller than 7μ. The significance of this state of affairs is not yet clear; evidently special factors are at work in the morphogenesis of this nerve.



Text-fig. 2. Internodes from lateral line nerve of a small specimen of *Conger* (63 cm. in length).



Text-fig. 3. Internodes from electric organ nerve of *Torpedo*.

In the nerves of the largest fishes investigated, in the region of the smaller fibres the internodes do not increase in length with the diameter. This is shown in Text-figs. 4, 5 and 6, which represent internodes from the lateral line and branchial nerves of the 83 cm. ray, and the lateral line nerve from the 124 cm. conger eel. Above 5μ the relationship is approximately linear and Text-figs. 4, 5 and 6 show regression lines fitted to all fibres larger than 5μ. The significance of this divergence is discussed on p. 345; it shows that internode length does not always increase with diameter.

(4) *The relationship between internodal length and the length of the fish*

This comparison was made using the lateral line nerves of fishes of different sizes. The total length of the fish was used in preference to the actual length of the nerve. To ascertain whether there exists a correlation between body length and internode length, the ratio of the value of the internodal length at the maximum fibre diameter to the length of each fish was employed. The results in the case of two conger eels are set out in Table 1. The standard error is given as a measure of the variance of the mean value of the internodal length at the maximum diameter. It will be seen that each ratio falls within the standard error of the other. Thus it may be stated that for these two nerves

$$\frac{ym}{L}=k.$$

The lateral line nerves from three rays yield the data shown in Table 2.

Here the ratio is not quite constant, the internodes of the fibres of the largest fish being slightly but significantly longer than expected.

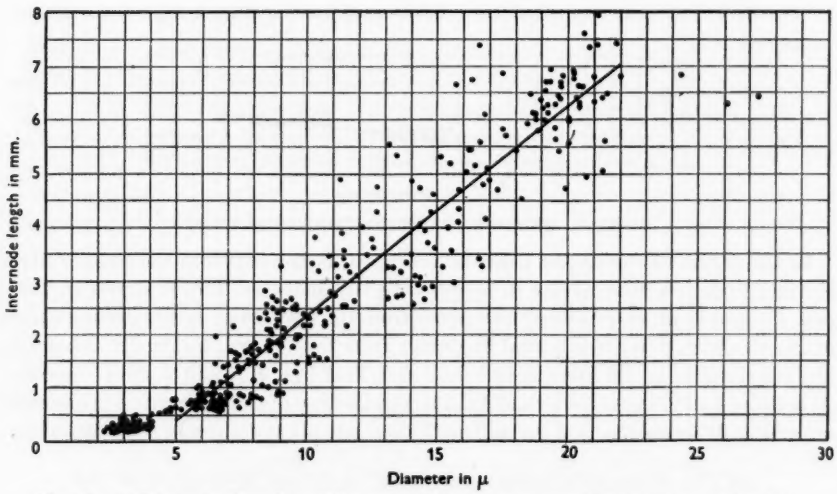
Somewhat similar measurements are given for the ray by Ranvier (1872); these are set out in Table 3. He recorded 'maximum internodal length' and the length of the fish. The ratio of the former to the latter has been calculated in each case and is

Table 1. *Relationship between internode length and length of fish in the conger eel*

	Length of fish, L (cm.)	Internodal length at max. diam., ym (mm.)	Int. length at max. diam. / length of fish
1	124	$7{\cdot}52 \pm 0{\cdot}20$	$0{\cdot}0606 \pm 0{\cdot}00165$
2	63	$3{\cdot}84 \pm 0{\cdot}11$	$0{\cdot}0609 \pm 0{\cdot}00174$

Table 2. *Relationship between internode length and length of fish in the ray*

	L (cm.)	ym (mm.)	$\frac{ym}{L}$
1	83	$7{\cdot}02 \pm 0{\cdot}09$	$0{\cdot}0845 \pm 0{\cdot}0011$
2	40	$2{\cdot}89 \pm 0{\cdot}04$	$0{\cdot}0722 \pm 0{\cdot}0009$
3	34	2·47	0·0726



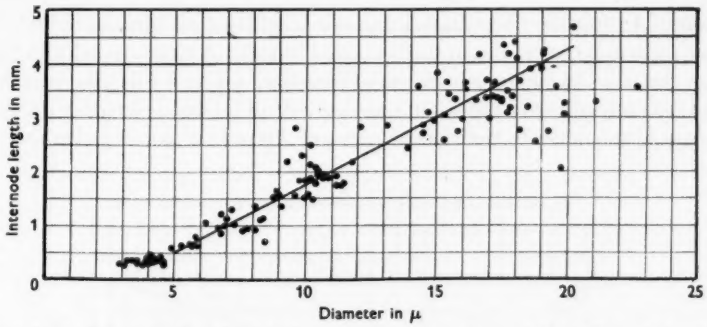
Text-fig. 4. Internodes from lateral line nerve of a large specimen of *Raia* (83 cm. in length).

approximately constant. The ratios are somewhat lower than those which we have found in *Raia clavata*; this may possibly be a species difference, but unfortunately Ranvier does not state which particular ray he investigated. Alternatively, a different region of the nerve might have been examined.

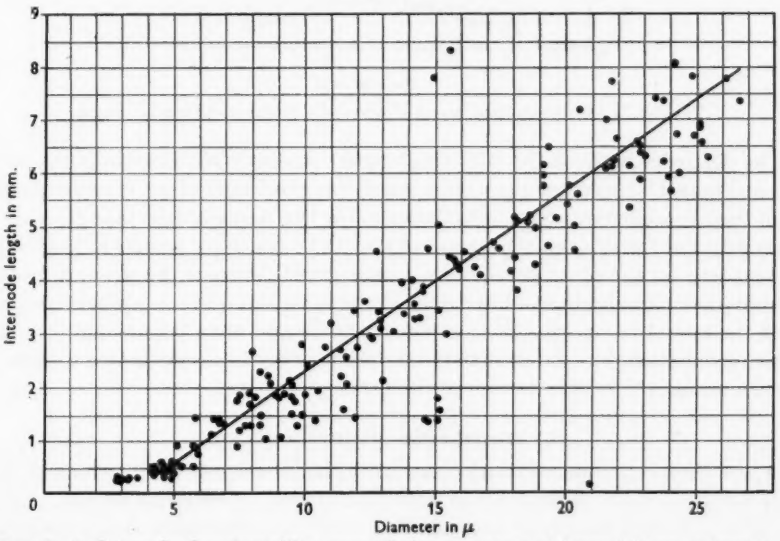
Comparison of maximum internode length with total length certainly supports the hypothesis put forward by study of the nerves of growing mammals that the internodal length is determined by the amount of length increase after the myelin has been laid down. The great length which the internodes may attain in fishes is presumably due to the relatively great amount of growth occurring subsequent to medullation.

(5) *Internode lengths in different nerves of the same animal*

If growth influences the distance between nodes, then differences should appear between nerves in regions of any one animal growing more, or less, in length, after medullation. Text-figs. 6 and 7 show the internode lengths in the lateral line nerve



Text-fig. 5. Internodes from branchial nerve of a large specimen of *Raia* (83 cm. in length).

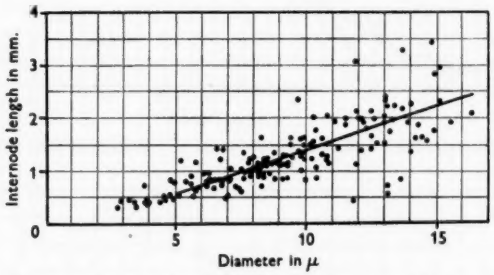


Text-fig. 6. Internodes from lateral line nerve of a large specimen of *Conger* (124 cm. in length).

Table 3. *Relationship between internode length and length of fish in the ray (data from Ranvier, 1872).*

	Length of fish, L (cm.)	Max. int. length (mm.)	Max. int. length / L.
1	110	6	0·0545
2	101	5	0·0495
3	36	2·5	0·0694
4	24	1·5	0·0625

and a branchial branch of the vagus of *Conger conger*. It must be remembered that the nerves contain functionally very different fibres and perhaps for this reason the maximum diameters reached differ greatly; no fibres in the branchial branch exceed 16 μ. However, it is obvious that internode length increases faster with diameter in the lateral line nerve (Text-fig. 9*A*): the slopes of the regression lines are given by $b = 0{\cdot}32 \pm 0{\cdot}0002$ and $0{\cdot}16 \pm 0{\cdot}0002$; the difference is highly significant, the value of t being 8·09. At 16 μ the fibres have internode lengths of 4·2 mm. in the lateral line nerve as against only 2·4 mm. in the branchial branch. A similar condition has been found in the same two nerves in the ray (Text-figs. 4, 5 and 9*B*); the gradients are 0·39 and 0·24 respectively. The slight increase only of internode length with diameter in the case of the nerve to the electric organ of *Torpedo* is possibly also an instance of this.



Text-fig. 7. Internodes from branchial nerve of a conger eel 124 cm. in length.

The difference between the internode length—diameter relationship in the lateral line and branchial nerves can be considered as being the result of one of two sequences, or of their combination: first, medullation in the two nerves might take place simultaneously and the subsequent amount of growth be relatively greater in length than in breadth; on the other hand, with similar growth rates in length and breadth, medullation might begin at a later stage in the branchial nerve as a whole compared with the lateral line nerve. Both explanations depend upon the amount of growth after medullation, but unfortunately no data suitable to decide between them are known to us.

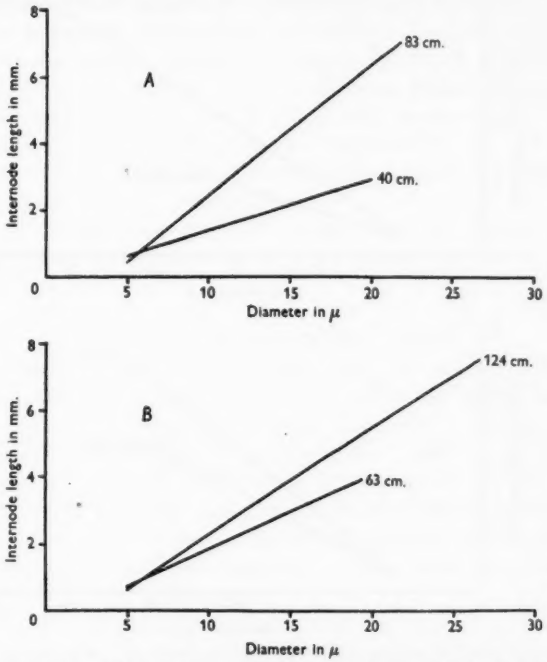
(6) *Irregularities of internodal length*

Apart from continuous variation on either side of a mean value, which at times may be considerable (for instance in Text-fig. at 16·6 μ the range is 4·02 mm.), certain gross irregularities have been noticed. Frequently the internodes are approximately twice or half the mean for the particular diameter; such are well illustrated in Text-fig. 6. These phenomena were also observed by Vizoso & Young (1948) and interpreted as being due to the disappearance of a node in the case of the long segments, and the formation of short internodes after regeneration. Occasionally very short internodes (0·2 mm. as in Pl. 1, fig. 7) were found intercalated between longer ones (4·4 and 4·0 mm.). These resemble the intercalated segments of Renaut (1881) which Vignal (1883*b*) supposed to be a normal feature in the histogenesis of nerves. Such irregularities seem, however, to be commoner in the older specimens, as indeed was

noticed by Renaut himself, and hence are more probably degenerative in origin (see Duncan, 1930; Corbin & Gardner, 1937; Rexed, 1944). The figure 0·2 mm. is interesting since it is that commonly found for newly formed segments. Fibres with alternately long and short internodal segments, as described by Kubo & Yuge (1938) in the toad, have not been observed.

(7) *The origin of the correlation of internodal length with fibre diameter*

It was early recognized that internodal length is greater on larger diameter fibres than on smaller (Ranvier, 1875; von Kölliker, 1896); Key & Retzius (1876) found this relationship in the dog, rabbit, finch, pike and man and it has been demonstrated for



Text-fig. 8. Regression lines of lateral line nerves of: *A*, specimens of *Raia* 83 and 40 cm. in length; *B*, specimens of *Conger* 124 and 63 cm. in length.

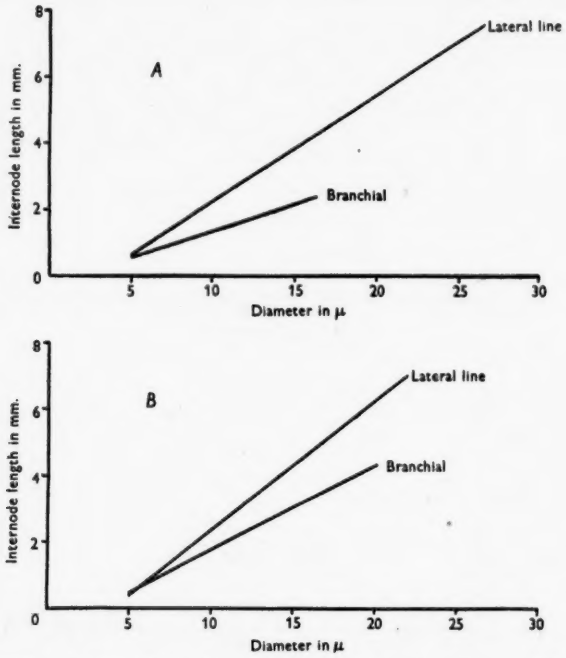
normal nerves in a more exact manner by all subsequent inquiries. Boycott (1904), Takahashi (1908) and Hatai (1910) found it to obtain in the frog, Kubo & Yuge (1938) record the same in the toad, rabbit, cat and man, Hiscoe (1947) in the rat, Vizoso & Young (1948) in the rabbit.

Our present study on fishes shows a linear increase of internode length with fibre diameter in nerves of various fishes, but with the exception of the smaller fibres in the nerves of large animals, which all have similar and short internodes (p. 339). Moreover, where the internode length does increase with diameter, the relationship may vary greatly, for instance between young and old animals (p. 339 and Text-fig. 8), or between different nerves of the same animal (p. 341 and Text-fig. 9). Clearly,

therefore, internode length is not solely a function of diameter, a result also strikingly shown in mammals by the fact that after regeneration of a nerve the internodes are all short (Leegaard, 1880; Young, 1945*b*; Hiscoe, 1947; Vizoso & Young, 1948), although the normal diameter spectrum is re-established (Gutmann & Sanders, 1943).

Presumably the proportionality found in normal adult animals between length of internode and fibre diameter is the result of correlation of both with some common factor. We must therefore search for correlation of each with other variables.

The fact that after nerve regeneration in an adult the internodes are short on fibres of all diameters suggests that growth is the factor which controls internode length.



Text-fig. 9. Regression lines of lateral line and branchial nerves from: *A*, a conger eel 124 cm. in length *B*, a ray 83 cm. in length.

(8) *Correlation of internodal length with growth of nerves*

Ranvier was quick to draw attention to the fact that internodal length is greater in larger than in smaller specimens of an animal. He arrived at this conclusion from measurements carried out on the rays (1872) and the dog and rabbit (1875). This relationship was corroborated by Boycott (1904), Takahashi (1908) and Hatai (1910). The two latter workers noted that internodal length in the nerves to the hind-limb of the frog increases distally, and thought that this was associated with the greater growth rate of the shank as compared with the thigh.

Similar observations have been made by Hiscoe (1947) in the rat, Vizoso & Young (1948) in the rabbit, and now by ourselves in fishes. In the lateral line nerves

examined, internode length was found to be approximately proportional to the amount of growth that the animal had undergone (p. 340).

It has been noticed by several investigators that the gradient of the line relating internode length to diameter increases with gain in size of the animal; that is, that the increase of internode length with growth is relatively greater than that of diameter. Vizoso & Young (1948), from data obtained from the peroneal nerves of growing rabbits, stated that the slope of the regression lines increased in the same proportion as did femoral length. In the present study, we have found the gradient to be greater in the larger animals (Text-fig. 8).

The differences between the internode lengths in the lateral line and branchial branches of the vagus nerve may be, as already suggested (p. 342), a function of the relative amounts of growth in length that they undergo. Again, a similar phenomenon might provide an explanation for the constancy of internode length in the smaller fibres of the largest fishes investigated. These fibres are presumably those that have medullated most recently (see later, p. 346). If this process occurs throughout the life of the animal, those fibres that become myelinated during the period of the decline in growth rate may increase in diameter in the same way as their predecessors, but fail to undergo the same increase in length.

Thus, although the data about growth rates are incomplete, the facts suggest that internodal length is strongly correlated with the amount of growth of the part in which the nerve lies.

(9) *The determination of fibre diameter*

If internode length is correlated with growth, can this explain its correlation with fibre diameter? Unfortunately we know very little of the factors that determine diameter, but they are likely to be complicated, since each functional fibre type has its characteristic diameter. Diameter is certainly not highly correlated with length: Schwalbe (1882) supposed the longest fibres to be the largest, but Dunn (1900, 1902 and 1909) showed that in the frog, the longer fibres are, if anything, smaller. Several lines of evidence, however, show that not all fibres medullate at the same time, and if the ultimately larger ones medullate before the smaller there might be an approximate correlation of diameter with growth.

Numerous investigators have found that the appearance of myelinated fibres is prolonged over a considerable part of the growth period in mammals (Hatai, 1902, 1903; Boughton, 1906; Duncan 1934*a*; Corbin & Gardner, 1937; Kjellgren, 1944; Rexed, 1944). On the other hand, Schiller (1889) observed only a very slight increase in the numbers of myelinated fibres in the oculomotor nerve of the cat between newborn and adult animals; but there is reason to suspect that the disparity is the result of the less satisfactory technical methods available to him. Birge (1882) and Hardesty (1899, 1900) have obtained similar results in the frog.

There is also some evidence of a correlation between time of medullation and ultimate diameter reached by a fibre. Boughton (1906), Schimert (1935), Otuka (1910) and Rexed (1944) have all found that the fibres that appear later do not attain the diameter of their predecessors. Dissenting are Kiss & Mihálik (1930) who contend that the sympathetic preganglionic fibres in the ventral roots of man medullate before the eventually much larger motor fibres to voluntary muscle.

Thus it is probable that the eventually larger fibres become medullated before the smaller, although the detailed relationship has not been worked out. An approximate relationship would be sufficient to account for the correlation of fibre diameter and internode length, which is, after all, not a very close one.

(10) *Absence of increase of number of nodes after medullation*

The hypothesis here adopted implies that the number of nodes is constant for all ages except the most advanced, and this has already been suggested by several investigators. Nevertheless, Vignal (1883*b*) believed that intercalation of new internodes into nerve fibres takes place during growth, and Speidel (1932) has observed *in vivo* the end-to-end anastomosis of adjacent internodes with the obliteration of the intervening node. But Boycott (1904) came to the conclusion that in the frog the 'average internodal distance' increases in proportion to the increase in the length of the whole nerve during growth, and hence that there is no change in the number of nodes. The curve of the average internodal distance plotted against body length followed closely that of the length of the sciatic nerve against body length. This was later confirmed by Takahashi (1908). Hiscoe (1947) and Vizoso & Young (1948) reached essentially the same conclusions in the rat and rabbit respectively. We may assume for the present that in fishes, as in amphibians and mammals, the number of internodes does not increase once they have been laid down, but it would be desirable to have direct evidence of this, especially since the internodal segments are so long in fishes and each may contain several Schwann nuclei.

(11) *When medullation takes place internodal lengths are the same in all fibres*

One of the most striking results emerging from various studies is that there is a minimum internode length that is approximately the same from fishes to mammals. Vignal (1883*a*) reported this to be 0·2 mm. in a foetal sheep. Young (1945*b*) reported it as 0·15 mm. in embryo rabbits, but this may be a little low. Vizoso & Young (1948) found no fibres with internodes below 0·25 mm. in the 2-week-old rabbit. Hiscoe (1947) gives 0·33 mm. for fibres 2·6–4·5 μ in rats 2–4 weeks old, but the nodes may well be a little shorter at the time of medullation in the foetus. Similarly, Ranvier (1875) recorded that the internode length on the largest fibres of the sciatic nerve of the newborn dog is about 0·3 mm.

In the present investigation there is a very sharply demarcated minimum to the internode length data. Thus in Text-fig. 4 it is remarkable that no internode is less than about 0·20 mm. long, the strictness of the determination strongly suggesting the influence of a uniform physical condition. Essentially similar conditions are seen in the younger ray, in *Torpedo*, and in *Conger*.

These facts lend support to the view that this initial periodicity is determined by a limiting physical condition, such as the stable length of a droplet under surface tension (Young, 1944, 1945*a*, *b*).

Speidel (1932, 1933 and 1935) indicated that the Schwann cells become spaced at approximately regular intervals and that the myelin first appears in the region of their nuclei. Hiscoe (1947) suggested that it is this spacing that is the determining factor and cited in support the observation of Weiss & Wang (1945) that 'the basic length of the Schwann cell in tissue culture is between 200 and 500 μ', this length

coinciding roughly with the internodal distances of the rats 2-4 weeks old that she investigated. The early development of fish nerves requires elucidation, for whereas in the other vertebrates studied there is but one Schwann nucleus per internode, usually situated near the middle, in the fish nerves we have examined there may be several. Attention has been drawn to this by Key & Retzius (1876). A comparison of the number of nuclei per internode with internodal distance might yield interesting results; the data of Key & Retzius concerning this are too fragmentary to allow of any definite conclusion. In the regenerated nerves of mammals there are also several nuclei per internode.

CONCLUSIONS

Abundant facts are available about lengths and diameters of nerve fibres: few tissues have been so satisfactorily studied by quantitative methods. The figures obtained are all consistent with the view that myelin is laid down on young nerve fibres when they reach about 1–2μ in diameter (Duncan, 1934*b*; Schmitt & Bear, 1937). This liquid layer does not remain continuous over the fibre, but breaks up into a series of myelin segments, each about 0·2 mm. long. A neurilemmal tube is rapidly formed around the myelin, so as to make a chamber containing the liquid.

Normally these myelin segments remain throughout life, each fibre retaining the number of nodes originally laid down. Occasionally internodes may fuse, by disappearance of the intervening neurilemmal inflexions, and also when the axon degenerates and regenerates the original periodicity is lost.

During growth each segment becomes stretched by the tension exerted by neighbouring parts. The neurilemmal sac is closed and more myelin is secreted between the Schwann cell and the axon surface. The length reached by any internode therefore depends on the amount of growth in length of the nerve after it has been laid down.

Since fibres that will ultimately be large are in general laid down early, a relationship appears in a normal adult between diameter and internode length, the latter rising more steeply with diameter in the nerves of those parts of the body in which a relatively greater amount of growth occurs subsequent to medullation.

If the final internode length reached is a function of growth, it is difficult to believe that it is an important variable in controlling conduction velocity or any other functional feature as supposed by Hursh (1939) and von Muralt (1946). The nodes may be fundamentally important as points of high permeability, but their exact spacing seems to have little importance for function. The experiments of Berry, Grundfest & Hinsey (1944) and Sanders & Whitteridge (1946) confirm this by showing that in regenerated nerves conduction velocity increases with the diameter of fibres, although internode lengths remain short.

SUMMARY

1. In the lateral line nerves of the ray and conger eel the distance between the nodes of Ranvier may reach 8 mm. on the largest fibres.

2. Internode length increases greatly with fibre diameter in the long lateral line nerves, but in the shorter branchial nerves there is less increase and in the nerve of the electric organ of *Torpedo* the large fibres have only slightly longer internodes than the smaller.

3. In lateral line nerves from animals of different lengths the ratio of the largest internode length to the length of the animal is approximately constant.

4. There is not, therefore, a fixed internode length for each fibre diameter, the correlation between the variables is due to the fact that both are correlated with growth.

5. There is a sharply defined minimum internodal length of about 0·2 mm. in the various fishes studied, and this is also found in other animal groups.

6. Several Schwann nuclei may be found in each internode in fishes.

7. The conditions in fishes and other vertebrates are consistent with the view that internode length is determined by surface tension when the nodes are first formed and later by the amount of growth of the nerve. When medullation takes place internode length is short, and is the same for all fibres. As growth proceeds, a relationship between internode length and diameter appears, such that the larger fibres have longer and the smaller shorter internodes. There is little change in the number of nodes after medullation; as the nerves elongate, so too do the internodes. The occurrence of longer internodes on the larger fibres would be explained if the ultimately larger fibres became myelinated earliest.

The complete data, which are too bulky for publication, have been deposited at the Thane Library, University College, London. We wish to express our thanks to Dr F. S. Russell, F.R.S., Director of the Marine Biological Association, Plymouth, where part of this work was carried out; and also to Messrs D. I. Fryer, D. Sholl and A. D. Vizoso for advice and assistance.

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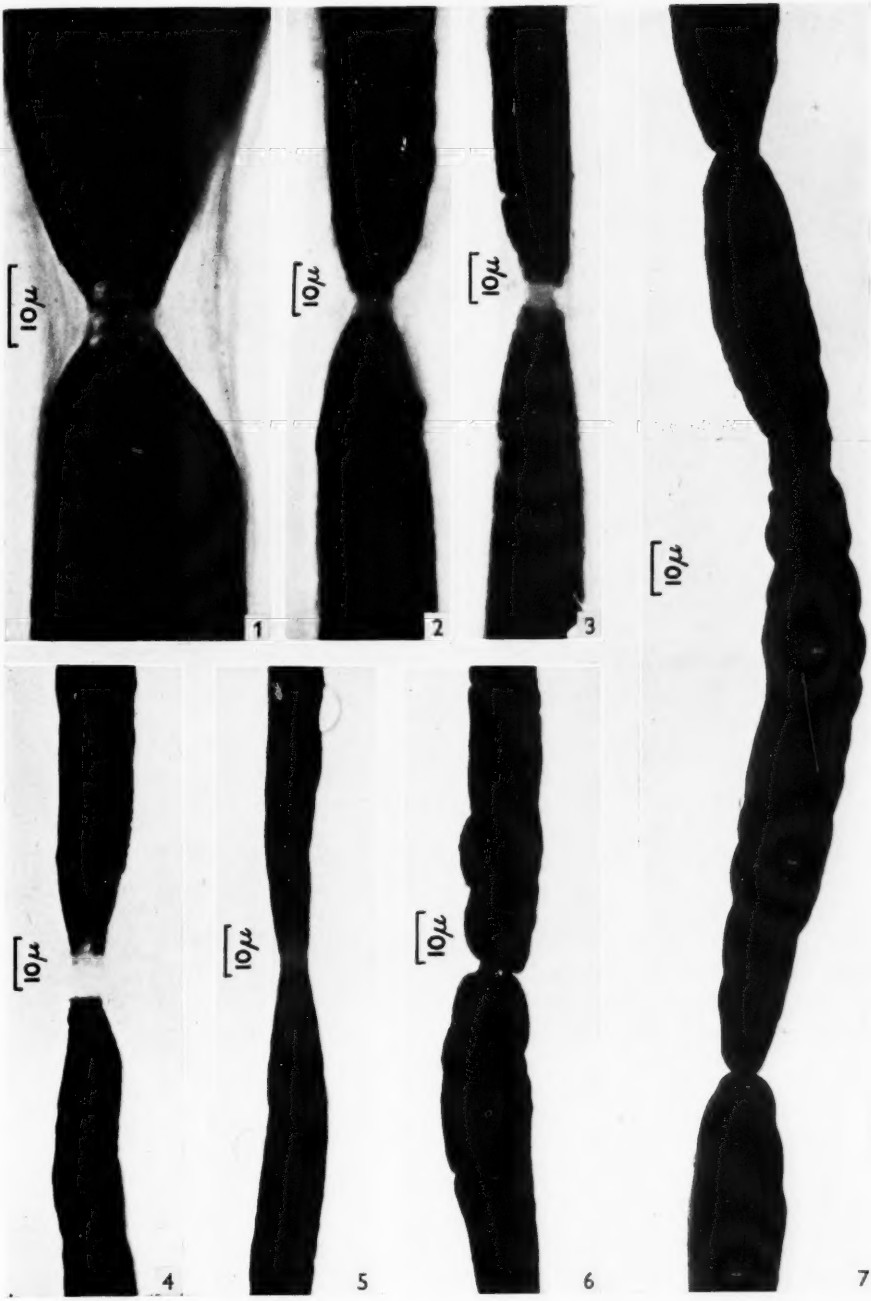
EXPLANATION OF PLATE

Photomicrographs of isolated nerve fibres stained with osmic acid.

Figs. 1–6: nodes of Ranvier in *Raia* (figs. 1–3), *Scyliorhinus* (fig. 4), *Torpedo* (fig. 5) and *Conger* (fig. 6).

Fig. 7: intercalated segment of Renaut in *Conger*.



THOMAS AND YOUNG—INTERNODE LENGTHS IN THE NERVES OF FISHES

DIFFUSION PHENOMENON COMPLICATING THE HISTOCHEMICAL REACTION FOR ALKALINE PHOSPHATASE

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INTRODUCTION

The histochemical method for demonstrating alkaline phosphatase in tissue sections, as devised by Gomori (1939) and Takamatsu (1939), has now been widely used for almost ten years, but some of the technical steps are not yet generally agreed upon and the evaluation of the results, particularly as regards precise cytological localization of the enzyme, is, in our opinion, still open to dispute.

Whilst most workers use a similar substrate mixture, either with or without added Mg-ions as enzyme activator, the time of incubation has been subject to wide variations, and many workers remark upon the increased intensity of the reaction that occurs on prolonging the incubation period. The time chosen for incubation would seem to be either quite arbitrary, or that which gives the optimum 'staining' result without loss of contrast between cytological details (Fell & Danielli, 1943; Dempsey & Deane, 1946; Wachstein & Zak, 1946). In other cases, the results of both a short and a long incubation period are recorded and the longer period (e.g. 24 hr. Fell & Danielli, 1943) given preference. The commonest period used is probably 2–3 hr. as originally advocated by Gomori (1939). The depth of 'staining' of the various structures is sometimes graded as black, dark-brown and light-brown and taken to indicate marked, moderate, and slight enzyme activity. If merely the intensity of the reaction were affected by a prolongation of the incubation period, no serious objection could be raised against this variation in the technique. It is, however, frequently stated that structures which are negative or almost so after a short incubation period, become definitely positive if the time of incubation is extended, and this seems particularly to be the case when they happen to lie in sections which somewhere have foci of high and fast reacting phosphatase content (e.g. Horowitz, 1942; Dempsey & Wislocki, 1947). This raises the important question of the localization of the enzyme. Does such delayed reaction signify a weak enzymic activity in these regions or is it due to some 'staining' effect or pseudo-reaction which is brought about by diffusion of some component or other from the true sites of enzyme activity? The existence of such diffusion is also suggested in the literature on the subject by numerous microphotographs—too numerous to be cited individually—which show an obvious gradient of positive cytoplasmic and/or nuclear reactions, often over a wide area, from foci of strong phosphatase activity. If, then, diffusion is at all involved in this histochemical reaction, it might also account for the well-known 'staining' of cells and nuclei (after the standard 2–3 hr. incubation period) at places close to sites of intense phosphatase reaction. Such instances are the internal structures of the epithelial cells of the small intestine or the nuclei of cortical renal tubules and glomeruli. A less familiar example can be observed in the rat pancreas where, not infrequently, the periductular connective tissue gives a strongly positive reaction

(Jacoby, 1946) and the adjacent duct epithelium shows 'stained' nuclei and a brownish granulated cytoplasm. Is one to speak here of moderate or weak phosphatase activity or of an artifact? The problem seemed to us of sufficiently fundamental importance to warrant investigation by specially designed experiments which might throw further light upon it.

Whilst this work was in progress the papers by Danielli (1946) and by Lison (1948) came to our notice, and both have an important bearing on this problem. Danielli has, in fact, performed experiments similar to, though not identical with, those to be reported here, and arrived at opposite results. He denied that diffusion plays any adverse part in the reaction and affirmed the reliability of the method with regard to the localization of the enzyme. Lison, on the other hand, in his exhaustive review of the histochemistry of phosphatases, frankly states that often some diffusion (i.e. of enzyme) obviously takes place which, in his opinion, is an artifact due to bad fixation.

In this paper experimental proof will be presented to show that diffusion does occur, and that it does so after and not during fixation. There is no evidence that it is connected with bad fixation in the histological sense.

MATERIAL AND METHODS

A. *General*

Except where stated otherwise, all tissues were prepared as follows. They were removed from the animals immediately after these had been killed by concussion and bleeding from the carotid arteries. Small pieces of the tissues, not exceeding a few mm. in thickness, were fixed in 80 or 95% alcohol (usually the latter), dehydrated in absolute alcohol, cleared in xylol, and impregnated with paraffin at 56° C. for 2 hr. The whole procedure was always completed within 24–30 hr. Sections were cut at a standard thickness of 7 μ, mounted on slides with a trace of egg-albumen and dried at 37° C., usually overnight.

The histochemical method employed for demonstrating the presence of alkaline phosphatase was a combination of Gomori's (1939) and Kabat & Furth's (1941) techniques, which was strictly adhered to except for the incubation period which was the main variable.

In view of the importance of the technique in a methodological study of this kind a brief summary of it seems indicated. The paraffin sections mounted on slides were treated in the following way: xylol 1 min., absolute alcohol 1 min., immersion in 0·5% celloidin (in ether-alcohol $\overline{aa}$) 2 min., hardening of the celloidin film in 90% alcohol 5 min., 70% alcohol, distilled water a few minutes. They were then incubated at 38° C. in the following substrate mixture: 3·2% sodium-β-glycerophosphate 6 ml., 2% calcium nitrate 9 ml., 10% sodium barbitone 6 ml., 0·1 M-magnesium sulphate 6 ml., and distilled water 33 ml. The pH of this substrate bath, as tested by means of a universal indicator, is about 9·4. After incubation and a brief rinse in distilled water the slides were placed in 2% cobalt nitrate for 5 min., washed in three changes of distilled water (occupying about 2 min.), immersed in dilute ammonium sulphide 2 min., washed in running tap water 1 min.; then dehydrated in the usual way, the celloidin film being dissolved in absolute alcohol or, more speedily, in an ether-alcohol mixture, cleared in xylol and mounted in Canada balsam. No counterstain

was used. Any deviations from this basic procedure which were necessitated for special tests will be referred to in the appropriate places.

Control sections were incubated in a 0·1 % calcium nitrate bath, the substrate being omitted; it may be said at this stage that in all instances these proved to be entirely and consistently negative.

B. *Special experimental procedures and results*

We developed two experimental approaches towards the problem at hand. The first consisted of an arrangement in which sections of known high phosphatase content were superimposed on sections known to be negative in this respect. It was thought that, if diffusion is at work in a section with a high enzyme content bringing about a positive reaction (or 'staining') in cells around the sites of high phosphatase activity, such diffusion should also affect a tissue which is placed underneath and that nuclei or cells—which are normally negative—could be made to appear to give a positive reaction.

Guinea-pig liver proved an ideal material to serve as underlying section. Apart from isolated positive leucocytes and/or litoral cells in and along vascular spaces, the liver of guinea-pigs gives no phosphatase reaction in its parenchyma (Pl. 1, fig. 1), at commonly employed incubation periods (i.e. up to 24 hr.). This holds true for paraffin as well as frozen sections. The fact that after excessively long incubation (70 hr.) the nucleoli of the liver cells sometimes show a faint 'staining', as Zorzoli & Stowell (1947) have also observed, does not detract from the value of this tissue for the present purpose. Furthermore, this very weak nucleolar reaction of the liver cell nuclei can be completely abolished by heating the section prior to it being used as underlying material.

The large polyhedral liver cells with their characteristic spherical nuclei constitute an easily recognizable tissue even when seen through a superimposed section.

Renal medulla (from rabbits) was also used for the same purpose; its parenchyma is well known to be histochemically phosphatase negative, and we have found that neither paraffin nor frozen sections prepared from it showed any nuclear reaction even after 120 hr. of incubation. But structurally renal medulla is not quite so suitable as guinea-pig liver for the purpose of these experiments.

Sections superimposed included duodenum, other parts of small intestine and mid-colon, and renal cortex of rabbits, and also small intestine and renal cortex of rats and of guinea-pigs. The heavy positive reaction given by the cuticular border of the small intestine and the convoluted tubules of the kidney need no further comment; but it is worth noting that rabbit mid-colon shows often a strong positive reaction in the tunica propria (affecting practically all the structures of this layer).

In detail, the procedure was as follows: First the 'negative' tissue was mounted and dried on a slide in the usual way. Then paraffin sections were cut from the 'positive' material, and small segments of them floated on to the 'negative' tissue. Care was taken to avoid much water accumulating between the two sections. The double-mounted slide was placed in the incubator and dried overnight. The phosphatase test was then performed in the manner described.

It was found with all these tissues that after an incubation period of 24 hr. or more

there was clear evidence of the occurrence of diffusion as reflected in the underlying negative sections. The nuclei of the underlying liver cells near sites of intense phosphatase activity in the superimposed section showed a typical 'positive' reaction. They were not jet-black throughout, but showed intensely blackened nuclear membrane, nucleoli and other chromatin particles (Pl. 2, fig. 6). There was a definite down-gradient from the sites of intense enzyme activity, so that liver cell nuclei some distance away from these sites showed no reaction at all (Pl. 2, fig. 5). The same result was obtained with rabbit renal medulla used as the basal negative section.

It was then thought possible, by varying the incubation period, to determine the time at which diffusion could first be detected, the criterion being a 'positive' nuclear reaction in the underlying tissue. Sections from each series prepared were incubated for periods of $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, 3, 6 and 18 hr. It was found that with enzymatically highly active tissue, such as the striated border of the small intestine, there could be detected a few 'positive' nuclei in the underlying section, very close to the border, in some instances at as short a period as $\frac{1}{2}$ hr. incubation (Pl. 1, fig. 2), but not invariably so (Pl. 2, fig. 7). At periods of 1 and $1\frac{1}{2}$ hr., diffusion was more clearly evident and more consistent, and became quite obvious and regular at the commonly used periods of 2 and 3 hr. (Pl. 1, fig. 3; Pl. 2, fig. 8). With times longer than this there was usually a further increase in intensity and spread of the 'positive' nuclear reaction in the underlying tissue (Pl. 1, fig. 4), gradually approaching a maximum which was sometimes reached at 18 hr. incubation.

With paraffin sections of renal cortex as superimposed material diffusion was weaker over the first 3 hr. than with small intestine (Pl. 3, fig. 9); whilst with rabbit colon an intermediate result was obtained; after $1\frac{1}{2}$ hr. of incubation many liver cell nuclei lying underneath the positive tunica propria of the colon showed a definite 'positive' reaction. The occurrence of diffusion with this colon material seems of particular interest and significance, for here the enzyme is obviously distributed and situated in a manner and at places different from what is found in the case of intestinal and renal epithelium. This observation therefore suggests that the phenomenon may, indeed, have a wider application.

Furthermore, it should be noted that in these experiments a close parallelism was often found between the appearance and degree of the 'positive' nuclear reaction in the underlying tissue and the spread of the reaction in the superimposed section itself. When, for instance, after 6 hr. incubation a superimposed section of small intestine showed positive nuclei throughout its wall, the nuclei of the underlying guinea-pig liver cells were found positive over a similar area, i.e. up to and including those that lay underneath the muscular coats.

The existence of diffusion having thus been established, the question arose whether its occurrence was in any way connected with one or other of the various steps of the method employed and whether it could perhaps be influenced by making certain alterations in the technical procedure. Of these, the covering with celloidin and its subsequent hardening were given special attention because, if the diffusing material was the enzyme itself, these steps seemed to be of particular importance. But neither varying the concentration of celloidin from 0·0 to 1 % nor the time of immersion in it (up to 30 min.), nor drying it in air (up to 15 min.) prior to hardening, nor varying the time of hardening in 90 % alcohol (5 min. to 6 hr.) affected significantly the result.

Diffusion occurred in all instances, but the impression was gained that drying of the celloidin film in air beyond 3 min. prior to hardening seemed slightly to reduce diffusion.

Omitting the Mg-ions from the incubation fluid, though somewhat weakening the intensity of the reaction and retarding diffusion, did not substantially alter the outcome; especially not after long incubation periods.

That the process of embedding was not responsible for the diffusion phenomenon was shown by employing frozen sections as superimposed material. Alcohol-fixed renal cortex and small intestine (from rabbit and guinea-pig) were used, some of which had been in absolute alcohol for 4 days, in order to ensure thorough fixation. The results were even more impressive than with paraffin sections, the reaction as a whole being more intense, and diffusion occurring readily and at shorter periods of incubation (compare, for instance, Pl. 3, fig. 10 with fig. 9). This is not surprising in view of the higher phosphatase content of frozen compared with paraffin embedded material (Danielli, 1946; Cappellin, 1947).

The possible influence of the time for which the tissue is left in paraffin (at 56° C.) during the embedding process was also tested. Two pieces of small intestine from a rat were used, one being left in the paraffin bath for 2 hr., the other for 18 hr. Sections from each were superimposed on guinea-pig liver and treated in parallel. The pictures obtained of the phosphatase reaction and the amount of diffusion produced appeared to be about the same in both cases; short and long incubation periods being studied.

Whilst examining the slides with sections of small intestine superimposed on guinea-pig liver or renal medulla, we had noticed that within the area corresponding to the lumen of the gut there were, here and there, amorphous masses showing a deep blackening, and areas of liver cells (or renal medullary tubules) underneath and around them also showing a positive nuclear reaction, fading towards the periphery. The contents of the small intestine are well known to be rich in phosphatase, and this would account for such a finding. It seemed, however, desirable to test this point by special experiments. Serial dilutions (with water), ranging from 1:5 to 1:500, were made from intestinal contents, fresh and alcohol-fixed; and a drop of each dilution smeared and allowed to dry on a section of guinea-pig liver or renal medulla. The phosphatase reaction was then performed. This brought out areas of liver cells (or renal medullary tubules) with a strong nuclear reaction which became weaker towards the periphery of such areas. The size and number of these areas decreased with the decreasing concentration of the film of intestinal contents. This experiment clearly shows that a positive nuclear reaction can be artificially produced by overlying phosphatase, and the gradient of the nuclear reaction strongly suggests a diffusion phenomenon.

Danielli, in his work already referred to (1946), has used as underlying sections rat kidney and spleen, and healing wounds (all containing phosphatase) which had been heated to destroy the enzyme, and frozen sections of alcohol-fixed kidney as superimposed material. He was unable to detect any diffusion in significant amounts. As his technique, especially the composition of the incubation fluid, differed slightly from ours, we applied his technical procedure to our material and repeated a whole series of the experiments, in some cases also heating the basic section for 40 min. at

110° C. Again diffusion was apparent in all instances, though the phosphatase reaction as a whole tended to be less intense, due, in all probability, to the absence of Mg-ions which are not included in the substrate mixture used by Danielli.

The second experimental approach was designed to find out whether certain parts of a section, which gave a positive reaction only after prolonged incubation, but which were suspected to do so as the result of diffusion from neighbouring areas of high phosphatase activity, would show a positive reaction, if first isolated by micro-dissection and then subjected to the histochemical test apart from the parent tissue. It was thought possible that such excised material would remain permanently negative and thus, in a negative way, provide further proof for the existence of diffusion.

For this purpose rabbit duodenum was used. With this tissue it had been found that often, on prolonging the incubation period, some or all the nuclei of Brunner's glands and even those of the outer muscle coats became positive; the degree and extent of the reaction varying somewhat from animal to animal. Sometimes only those acini of Brunner's glands that lay towards the lumen of the gut would show a positive reaction, a fact which in itself seemed to suggest a diffusion process.

For these experiments duodena were selected which showed such marked phosphatase activity that after 24 hr. incubation or longer nearly all the nuclei of Brunner's glands and the outer muscular coats as well as the usual sites showed a positive reaction (Pl. 3, fig. 12), whilst after short incubation periods the said nuclei were negative (Pl. 3, fig. 11). From paraffin sections of such material, floating in a small drop of water on a slide, peripheral wedges were cut out, under a dissecting microscope by means of a von Graefe knife, which were so chosen that they contained only a very few acini of Brunner's glands together with the adjacent muscle coats. Incidentally, these glands are easily recognizable in such floating waxed sections, appearing greyish black in transmitted light. The need for very small wedges will be obvious from reference to Fig. 11 of Pl. 3, which shows that, after only ½ hr. incubation, there can be found two sites of strong phosphatase activity: the inner border of the lining epithelium and patches of tissue amongst Brunner's glands. It is difficult to decide on the nature of this tissue, but it appears, in part at least, to be composed of capillaries. Inclusion of any such patches in the excised wedges might, naturally, obscure the results. However, by excising and testing such wedges in sufficient numbers, this danger can be avoided. The isolated wedges and the parent sections, from which they had been removed, were then mounted on separate slides, dried and treated in the usual way, wedge and 'donor'-section being incubated simultaneously in the same substrate solution.

Such suitably isolated wedges remained negative when incubated for periods ranging from 24 to 72 hr. (Pl. 4, fig. 13), whilst the parent sections and also whole neighbouring serial sections gave a generalized nuclear reaction in all tissues, including Brunner's glands and the outer muscle coats. Fig. 14 of Pl. 4 shows a reconstructed picture of a parent section with the wedge that had been excised from it and incubated separately.

ADDITIONAL OBSERVATIONS

Having found that diffusion may become evident after only ½ hr. of incubation, we thought it would be interesting to study the results of even shorter incubation periods. Without going into details of the various tissues thus investigated, it may suffice to put on record here that in the case of rabbit small intestine an incubation period of 2 min. only was enough to bring out a strong reaction of the striated border of the epithelial cells, the remainder of the section being entirely negative (Pl. 4, fig. 15).

A further observation was made on the duodenum (also of rabbit). Its covering epithelium commonly shows, in places, a positive reaction in the supranuclear region which, as Emmel (1945) has shown (especially in the mouse), is identical in position with the Golgi zone, and various suggestions have been made with regard to the functional significance of the presence of the enzyme here in relation to the supposed activity of the Golgi apparatus. We found that incubation for 20 min. produced a strongly positive reaction in the intestinal border of the epithelium and a weaker, but definite reaction in the Golgi region of many of its cells, whilst the nuclei and the remaining cytoplasm were practically negative at this period (Pl. 4, fig. 16). This finding might indicate that the Golgi zone has an inherent, independent phosphatase activity. Alternatively, it might suggest that its affinity for whatever diffuses from the heavily reacting border is slightly greater than that of the nuclei. In view of the ease with which nuclei can be made to give a positive reaction in the close vicinity of foci of high phosphatase activity, we are, at present, inclined to accept the positive reaction of this Golgi region as a genuine one.

DISCUSSION

The experiments described demonstrate that a process of diffusion interferes in the standard histochemical methods (Gomori-Takamatsu technique) used for showing the presence of the enzyme alkaline phosphatase, in such a way that it will produce an apparent positive reaction in structures, especially nuclei, that contain no inherent histochemically demonstrable alkaline phosphatase. Such diffusion is particularly favoured by the presence, somewhere in a histological section, of foci of high phosphatase activity. The longer the incubation period, the more likely is diffusion to occur, and our experiments indicate that, at least for certain tissues, the incubation periods commonly employed, i.e. 2–3 hr., are too long if precise localization of the enzyme is to be determined; whilst periods of 20–24 hr., which have been advocated must, at least for many tissues, be regarded as excessive from this point of view. If no more than an appraisal of enzyme activity is to be made, without the aim towards its exact localization, then there is no disadvantage in using long periods of incubation, in fact these might be favourable for making comparative estimates of enzyme activity in different tissues.

It will also have become apparent that an estimation of all sites of enzyme activity in a histological section is a difficult matter, and is perhaps in many cases impossible with the standard technique. Diffusion will first occur from the sites of most intense phosphatase activity with the result that not only inherently negative structures close to these sites might show an apparent positive reaction, but it may also affect and mask sites of weaker, but true enzyme activity. The latter sites may indeed need a longer incubation period to be revealed. They can in this way be evaluated,

but only if they lie well away from regions of intense enzyme activity and the intervening tissues show no reaction. The limitations of the method are thus obvious, and discretion must be exercised in an assessment of the localization of the enzyme. It is, for instance, open to doubt whether the nuclei of the lining epithelium of the small intestine have histochemically an inherent phosphatase activity, a point to which we shall return later.

To overcome or, at least, to reduce these difficulties we feel justified, if and when exact localization of the enzyme is aimed at, in recommending first of all omission of the Mg-ions from the incubation mixture, as is actually done by a number of workers; secondly, drying the celloidin film in air for 3–5 min. prior to hardening in 90 % alcohol, though this point needs further testing and confirmation; thirdly, to perform, as a preliminary experiment, such procedure as superimposing small sections of the tissue under investigation on paraffin sections of guinea-pig liver in order to determine the maximum incubation period which can safely be used without the result being complicated by diffusion.

In order to find out whether certain parts of a section have a weak enzyme activity that is being masked by diffusion, it might, in favourable instances, be possible to settle the point by an isolation experiment comparable with that which we have carried out on the duodenum, but it will not always be an easy technical procedure, and even micromanipulation may have to be resorted to.

Future tests of these kinds will reveal the range of the validity of our observation; for the number of tissues investigated so far has been naturally limited. But it is worth noting that in these tissues which showed the occurrence of diffusion, the enzyme is in all probability situated in extra- or near-extracellular sites. This holds for the striated border of the small intestine, the brush border of the convoluted renal tubules (see Danielli's remarks on this point, 1946), and probably also for the tunica propria of rabbit mid-colon and similar sites in other parts of the intestine where fine reticular fibres are usually amongst the first structures to react. In the loose connective tissue surrounding Brunner's glands the reaction is often related to blood capillaries, where it might be due to the phosphatase of the blood itself. On the other hand, there seem to exist sites containing the enzyme, under the action of which diffusion does not occur, or at least not readily. This is, for instance, the case with the individual cells in and along the sinusoids of guinea-pig liver which show an intense reaction; but there is no spread to the nuclei of the neighbouring liver cells. In these cells, most of which are probably leucocytes, the reaction is strictly intracellular, affecting predominantly the cytoplasm.

As to the pertinent question of what constitutes the diffusing material, whether the enzyme itself or its reaction product, our experiments do not permit of a definite answer. But some observations and considerations should be made on this question. Danielli, when putting this problem to an experimental test, assumed that calcium phosphate would be the diffusing material, as he had observed that nuclei have a high affinity for calcium phosphate when this is precipitated in a section by non-enzymic procedures. As already stated, Danielli in his experiments with superimposed sections did not observe such diffusion. We are unable to account for this, but it may be that his choice of material, especially renal cortex on heated renal cortex, may have been an unfortunate one and must have presented considerable difficulty in evaluation.

However, it must be added that in some cases Danielli had evidence of diffusion, but this occurred when the superimposed frozen material had come from 80% alcohol and had not been taken into absolute alcohol. The phenomenon was, therefore, considered to be due to diffusion of the enzyme itself from inadequately fixed tissue. This links up with Lison's contention, to which reference has already been made. Our material does not show signs of bad fixation in the histological sense. As to the fixation of the enzyme in tissue sections, little is known. Lison was the first of the histochemists to draw attention to the fact that the enzymes (i.e. phosphatases) are present in the tissues in a lyo- and a desmo-variety. He points out that it is only the latter which can be and is detected histochemically.

However, he does not make it clear which enzyme variety is involved in the diffusion phenomenon. There seem to exist, at least, two important possibilities: (*a*) The lyo-enzyme variety has not been completely lost in the process of fixation, embedding, etc., and such as is left behind gradually diffuses, whenever the material passes through watery solutions, into the surroundings where it becomes anchored on suitable surfaces or interfaces, especially such as are presented by cell nuclei; it then becomes active here and causes the precipitation of the phosphate. If this is the correct interpretation of the diffusion phenomenon, inadequate loss rather than bad fixation of enzyme would be responsible for it; and for precise localization an optimum loss of enzyme has to be aimed at—a rather precarious goal!

(*b*) If, on the other hand, the lyo-enzymes have, as is supposed, been completely eliminated prior to incubation, then the phenomenon needs another explanation. The desmo-enzymes, by definition, are held in their places. Here they are active and as a function of time and their own quantity produce increasing amounts of calcium phosphate. It is conceivable that not all the calcium phosphate formed will remain, or even find room, at the site of enzyme action, but that with time some will diffuse away and settle on other structures which have, for some reason or other, a high affinity for it. Nuclei, as Danielli has shown, are such structures. It is then visualized here in the usual way and produces the apparent positive nuclear reaction.

Menten, Junge & Green (1944*a*, *b*) have described a different technique for the histochemical demonstration of alkaline phosphatase by precipitating and visualizing the organic radical of a suitable substrate (calcium-β-naphthyl phosphate) in the form of an azo-dye. This method has been criticized because of its failure to demonstrate nuclear phosphatase in such epithelial cells as those of the convoluted renal tubules (Lorch, 1947). May it not be that, on the contrary, the enzyme localization with this method is more accurate and less complicated by diffusion? Perhaps the reaction product is here not easily adsorbed on to nuclear structures. In other words, these observations might be used for arguing that in the Gomori-Takamatsu technique the calcium phosphate—and not the enzyme—is the diffusing material.

Apart from these two main possibilities considered so far, a further alternative exists. It may be that many, if not all, nuclei actually contain the enzyme, but in an inactive form, and what diffuses is a substance counteracting this inactive state, either an activator itself or an inhibitor-removing substance. Such hypothesis is particularly attractive in view of the important role the enzyme is supposed to play in the nucleic-acid metabolism of nuclei. It would also solve a discrepancy which exists between the results of chemical analysis of isolated nuclei (see below) and

histochemical evidence with which we are concerned exclusively in the present investigation. Attractive as this idea is, our results with heated guinea-pig liver as underlying material, which still showed the diffusion effect, precludes its acceptance. It will, thus, be obvious that further experimentation is necessary to decide which of the various possible explanations holds true and will account for the diffusion phenomenon.

It is not within the frame of this paper to discuss the problem of nuclear phosphatase. On chemical analysis of isolated nuclei, as just indicated, the enzyme has been frequently, if not invariably, found, but the number of types of nuclei thus investigated is still restricted. On the other hand, it is certain that histochemically the resting nuclei of numerous tissues are consistently phosphatase negative. To what extent, then, can we trust histochemical evidence? The answer is usually given in the following form: a positive result shows the presence of the enzyme, but a negative result does not necessarily demonstrate its absence (Cappellin, 1947; Lison, 1948). In the light of our work this conception needs some amendment. Though a positive reaction doubtless indicates the presence of the enzyme, it does not necessarily give its precise localization, nay, may even mask histochemically inherently negative structures. As to a negative result, this may well demonstrate the absence of the enzyme under the conditions under which the tissue was examined. Such a negative result should be compared, as to its validity, not with the enzyme content of fresh tissue as assessed by chemical means, but with biochemical tests made on material treated in the same way as the section. Only a discrepancy in the latter comparison would invalidate a histochemically negative result.

Finally, the implication of this work with regard to the existing literature on the subject has to be briefly considered. It will be obvious that some work which has been done in the past will need some revising and amending. It is impossible to enumerate all the instances in the literature in which the results and interpretations are clouded by this diffusion phenomenon. In most cases positive nuclear reactions are reported without qualification, regardless of their situation in a section and possible vicinity to foci of high enzyme content. Only very occasionally the word 'apparent' has been added. Yet a few examples, chosen at random, might be instructive. Thus Horowitz (1942), investigating rat foetal heads with loci of high phosphatase activity, which are brought out within a few hours of incubation, states that, if incubated long enough, practically all tissues will show the presence of the enzyme. This, according to the author, agrees with chemical investigations which have shown that in most mammalian tissues the enzyme can be detected (but compare our earlier remarks on this point). Wislocki & Dempsey (1946), in their bone marrow studies, describe a positive reaction of the endothelium of vascular channels and of reticular fibres and also of the neighbouring (*sic!*) developing blood cells. The same authors (Dempsey & Wislocki, 1947), investigating various mammalian placentas, which give a strongly positive reaction at the border of the chorionic epithelium, report that the nuclei of the mesenchymal cells in the cores of the villi, though negative after 3 hr. incubation, are often positive in sections incubated beyond 3 hr. Again, Atkinson & Elftman (1947), in their interesting study on the increase of phosphatase activity in the mouse uterus under oestrogenic stimulation, remark that the enzyme content of the nucleus (i.e. of the epithelium) appears to

rise with the increment in cytoplasmic alkaline phosphatase; and also that, after prolonged stimulation, the amount of phosphatase in the periglandular stroma appears to be noteworthy.

The situation is more complex in such studies as Emmel (1946) carried out on the differential inhibition of alkaline phosphatase by potassium cyanide. He found, amongst other things, that the nuclear phosphatase of the intestinal epithelium (rat) is already inhibited at a lower concentration of KCN than that of the cuticular border (incubation period 3 hr.). There is as yet no definite evidence that these nuclei have histochemically an inherent phosphatase activity at all. They will always be positive at the commonly employed incubation period of 2–3 hr., and might well be so as the result of diffusion. If this is so, then Emmel's findings would not indicate a differential inhibition, but merely a graded suppression of the phosphatase at the border which, at the lower concentration of KCN, will itself still give a positive reaction, whilst its activity is sufficiently inhibited so as to prevent diffusion from occurring.

To establish whether or not these nuclei of the villus epithelium harbour histochemically an inherent phosphatase seems a rather important task. This can be illustrated from yet another context pertaining to the relationship of nucleic acids and alkaline phosphatase. Brachet & Shaver (1948) treated sections of alcohol-fixed mouse intestines with desoxyribonuclease. The nuclei then became unstainable with toluidine blue nor would they give a positive Feulgen reaction; but Gomori's alkaline phosphatase reaction remained very intense. The authors take this as evidence for the dissociation of desoxyribonucleic acid and phosphatase in support of Jeener's work (1946) on isolated red cell nuclei. Such evidence and interpretation can only be accepted if and when it will have been shown that diffusion from the striated border is not responsible for the 'positive' reaction given by the nuclei of the intestinal epithelium.

These examples, selected from the literature, may suffice to show the complications which arise as a consequence of this diffusion phenomenon occurring in this otherwise most valuable histochemical test for alkaline phosphatase; and their discussion might be helpful in indicating future lines of research.

SUMMARY

1. An investigation has been made to determine the reliability of the standard (Gomori-Takamatsu) histochemical technique in demonstrating the precise localization of the enzyme alkaline phosphatase.

2. Two different experimental approaches are described, the purpose of each being to determine whether or not diffusion occurs as a complication during the standard histochemical procedure.

3. It is shown that a process of diffusion does in fact occur during incubation of the sections, leading to an apparent positive result in structures that contain no inherent histochemically demonstrable alkaline phosphatase.

4. The degree and extent of the diffusion is roughly proportional to the incubation period and is often quite marked after the commonly employed incubation periods of 2–3 hr.

5. The implications of these findings are discussed.

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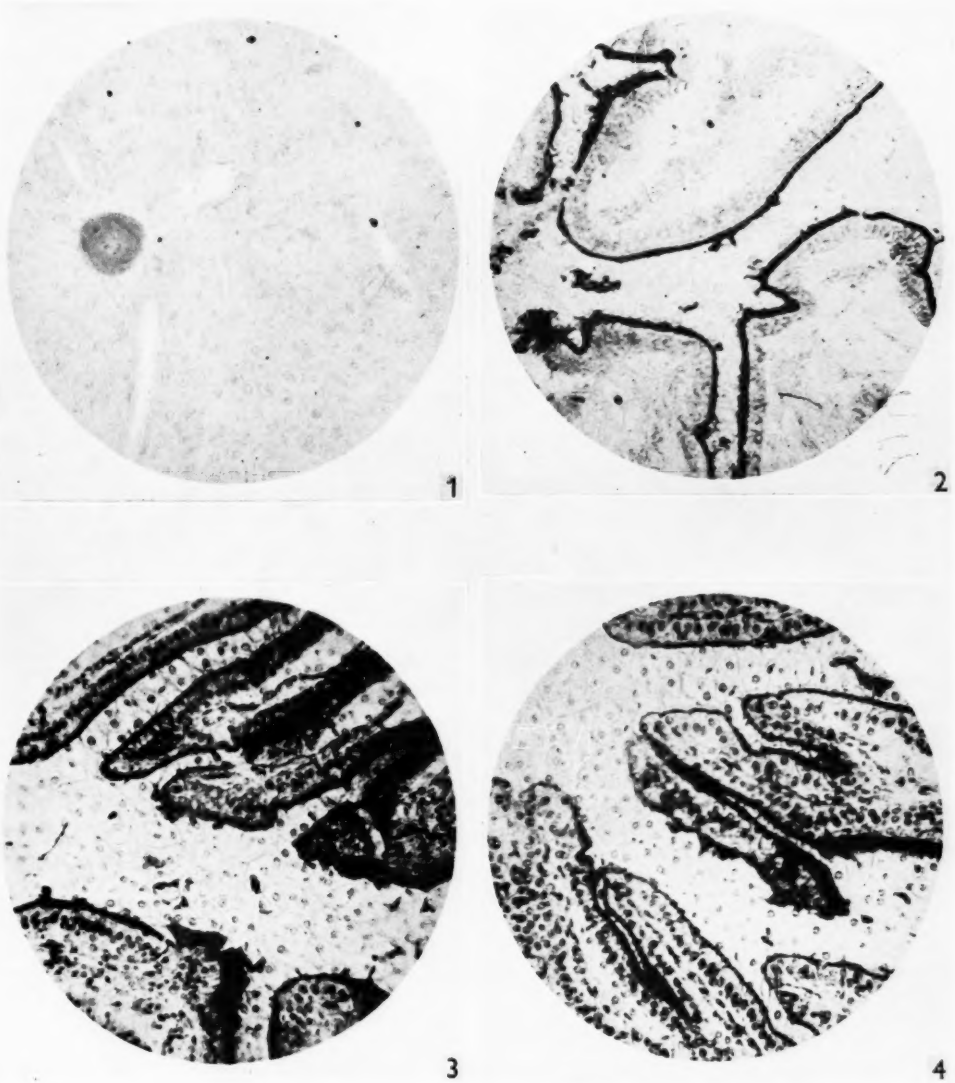
EXPLANATION OF PLATES

All figures are of sections which have been treated for alkaline phosphatase, sodium β-glycerophosphate being used as substrate. No counterstain was used.

The double-mounted sections on being photographed presented some focusing difficulty, and either an intermediate focus had to be chosen or preference had to be given to a sharp focus for the nuclei of the underlying section. This has resulted in a slight blurring in some of the photographs, affecting mainly the superimposed section.

A Wratten G Filter was used throughout.



MARTIN AND JACOBY—DIFFUSION PHENOMENON IN ALKALINE PHOSPHATASE REACTION

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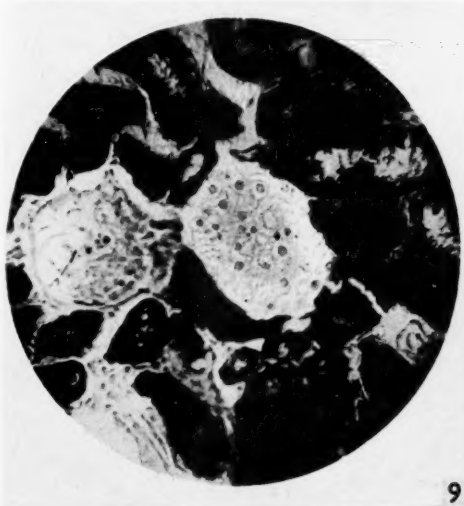

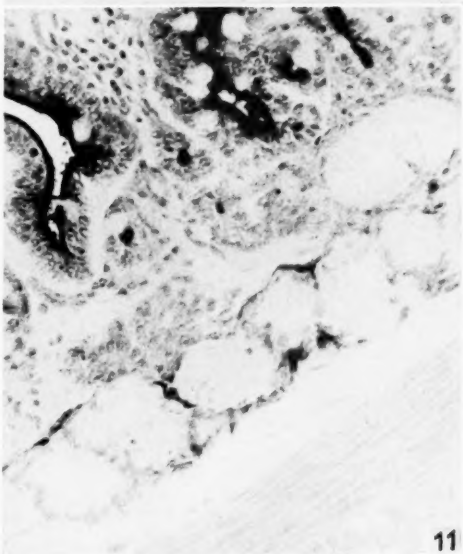

MARTIN AND JACOBY—DIFFUSION PHENOMENON IN ALKALINE PHOSPHATASE REACTION

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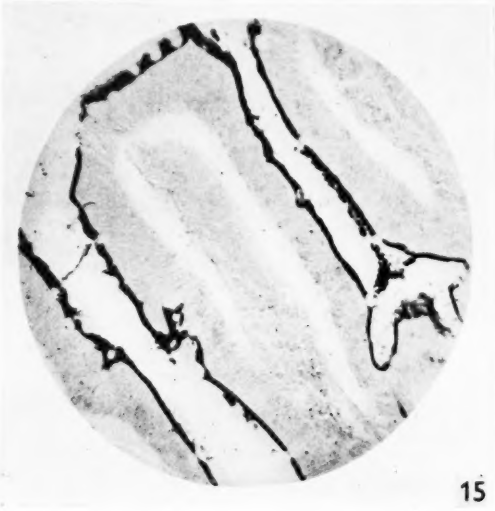
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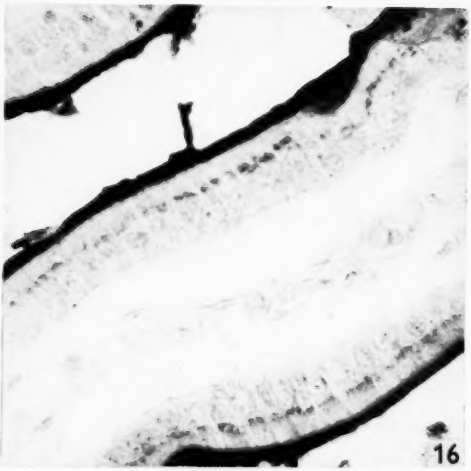
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MARTIN AND JACOBY—DIFFUSION PHENOMENON IN ALKALINE PHOSPHATASE REACTION

PLATE 1

Fig. 1. Guinea-pig liver. 24 hr. incubation. Note that except for a few blackened isolated cells in sinusoids the tissue is entirely negative. × 190.

Fig. 2. Rabbit duodenum on guinea-pig liver. ½ hr. incubation. Strong reaction of the striated border, of some intestinal contents, and again some positive cells in the liver sinusoids. The nuclei of the intestinal epithelium and also some liver cell nuclei (upper left-hand corner) are just starting to give a faint positive reaction. × 190.

Fig. 3. Rabbit duodenum on guinea-pig liver. 3 hr. incubation. Note in this and the following three photographs the increasing reaction given by the nuclei of the intestine and the underlying liver cells. × 190.

Fig. 4. Rabbit duodenum on guinea-pig liver. 6 hr. incubation. Note decreasing gradient of the hepatic nuclear reaction towards the upper left-hand corner. × 190.

PLATE 2

Fig. 5. Same preparation as that of Fig. 4. × 130, to show the gradient more clearly.

Fig. 6. Rabbit duodenum on guinea-pig liver. 24 hr. incubation. × 190.

Fig. 7. Rabbit duodenum on rabbit renal medulla. ½ hr. incubation. All nuclei practically negative. × 190.

Fig. 8. Rabbit duodenum on rabbit renal medulla. 3 hr. incubation. Note strong nuclear reaction not only in the intestine, but also in many of the underlying renal tubules near to the intestinal border. × 190.

PLATE 3

Fig. 9. Rabbit renal cortex (paraffin section) on guinea-pig liver. 3 hr. incubation. Note faint, but definite positive nuclear reaction in the liver cells in the centre of the photograph. × 190.

Fig. 10. Rabbit renal cortex (frozen section) on guinea-pig liver. 3 hr. incubation. Note strong positive nuclear reaction of the underlying liver cells. Compare with Fig. 9. × 190.

Fig. 11. Rabbit duodenum. ½ hr. incubation. Brunner's glands and outer muscle coat practically negative. But note the few positive connective tissue patches between the glands. × 280.

Fig. 12. Rabbit duodenum (same tissue as shown in Fig. 11). 48 hr. incubation. Note heavy positive nuclear reaction in Brunner's glands and also in the muscle. × 280.

PLATE 4

Fig. 13. Excised wedge from section of rabbit duodenum, containing Brunner's glands and outer muscle coat (same material as shown in Figs. 11 and 12). 72 hr. incubation. Result: reaction entirely negative. × 280.

Fig. 14. Reconstructed picture with excised wedge and parent section. Both were incubated simultaneously, but separately for 24 hr. × 200 approx.

Fig. 15. Rabbit small intestine. 2 min. incubation. × 190.

Fig. 16. Rabbit duodenum. 20 min. incubation. × 580.

THE DEVELOPMENT OF MOUSE HAIR *IN VITRO* WITH SOME OBSERVATIONS ON PIGMENTATION

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INTRODUCTION

In the past much attention was paid to the development and structure of hair, and more recently interest has turned to the effects of nutrition and climate on hair growth. Although very many factors have been shown to affect hair growth, little is known of their mode of action, and a number of reports are conflicting. There is need for further study of the interplay of heredity and environment and for an analysis of systemic and local effects on hair differentiation and growth.

Skin grafting has been used by many workers to study the physiology of hair growth. Reed & Sander (1937) employed the special technique of grafting embryo mouse skin on to newborn mice. Butcher (1946) transplanted pieces of the skin of young rats either subcutaneously or into the peritoneal cavity of the donor animals without interrupting the vascular or nervous supply.

There have been several attempts to alter the environment of hair follicles more thoroughly by making grafts to the developing chick. Hiraiwa (1927) showed that pieces of rat embryos underwent differentiation on the chorio-allantoic membrane of the chick, and claimed that normal hair follicles were developed. However, Nicholas & Rudnick (1933), using the same technique, were unable to confirm his results for epidermal structures. Reed & Alley (1939) sometimes obtained differentiation of hair follicles from embryonic and newborn mouse skin on the chick chorio-allantois. Normal hair follicles differentiated in grafts of embryonic mouse tissues in the chick coelom made by Rawles (1940, 1947).

Hair follicles were reported by Waterman (1940) in some of his grafts from mouse embryos to the omentum of the adult rabbit. Grafts of rabbit and rat embryo tissues to the adult rat kidney and to the adult rabbit kidney and omentum were much less successful than grafts to the chorio-allantoic membrane of the chick (Waterman, 1936).

All of the above techniques expose the skin and hair follicles to a blood-vascular system in which the volume and composition of the nutrient fluid is continually changing. They are therefore unsuited to analytical studies of the effect of nutrition and environment on hair development and growth, although they have proved very useful for other purposes as in the study of pigmentation and some hereditary factors.

The object of the present work is therefore to develop a method for studying hair differentiation and growth in the absence of a circulatory system. Such a method could be used to examine the effect of nutrition, temperature, light and other radiations acting directly on the skin, without confusion from systemic effects. To the knowledge of the writer only three attempts have been made previously

to grow hairs *in vitro*, and of these only two have been published as short notes. Strangeways (1931) obtained some differentiation of hair follicles when the skin of embryo guinea-pigs was grown in tissue culture, but she was more successful with the vibrissae of the snout than with ordinary pelage hairs from the trunk. Murray (1933*a*,*b*) made some unpublished observations on hair-follicle differentiation *in vitro* from embryo rat skin, and found that while vibrissae developed normally, the follicles of pelage hairs were unable to do so. Rawles (1940) referred in a footnote to a few trial tissue cultures of embryonic mouse skin made by Mr H. L. Hamilton, and reported that hair follicles differentiated, but gave no details.

The present paper describes the complete differentiation of hair follicles and the growth of hair in explants of embryonic mouse skin cultivated *in vitro*.

MATERIAL AND METHODS

Most specimens of the house-mouse, *Mus musculus*, were from the laboratory stock of albinos, two were from a mixed stock with grey-bellied agouti, or 'wild-type' colouring, and one was a mouse of the C57 Black strain. Embryos from 10 to 18 days post-coitus were used. The ages were calculated to the nearest day from the time of mating, indicated by the vaginal plug, and were checked by Grüneberg's (1943) description of the external features. In the case of embryos of 13 days or more, the vaginal bleeding served as an additional check. One, two or three members of a litter were used in each experiment, and pieces of the skin were fixed for controls.

The culture medium consisted of equal parts of fowl plasma and chicken-embryo extract. The extract was prepared by adding two volumes of Tyrode solution to one volume of a minced chicken embryo between 10 and 12 days old. The explants were transferred to fresh medium every 3–6 days.

Nine series of hanging-drop cultures were made from the dorsal and lateral surfaces of the trunk, and occasionally the ventral surface also was used. With the aid of a low-power binocular microscope, a sheet of ectoderm with the underlying mesoderm was removed from the body with fine cataract knives. This sheet was cut into squares with a side of about 1·5 mm., and each square was placed on the surface of a clot of plasma and extract, with the mesoderm next to the clot. The cultures, on $1\frac{1}{4}$ in. square cover-slips, were inverted over $3 \times 1\frac{1}{2}$ in. hollow-ground slides and incubated at 34 or 37°C. Living cultures were examined under the microscope; individual follicles could be clearly seen and their progress recorded from day to day. It was often possible to distinguish the individual cells composing a follicle and to follow their differentiation.

In the three series of cultures made by the watch-glass technique, larger pieces of tissue were cultivated in a greater volume of the medium than was used for the hanging-drop method. In Exps. 10 and 12 each explant consisted of the dorsal, lateral or ventral skin of one side of the trunk, and frequently included also portions of the underlying ribs, sternum or vertebrae. In the cultures from 10-day embryos of Exp. 11 each explant consisted of the entire dorsal or ventral half of the trunk. The watch-glass cultures, which were incubated at 37°C., were examined daily; the general state of hair follicle development was noted, but details could not be seen as clearly as in the hanging-drop cultures.

Cultures were fixed at intervals throughout each experiment, and usually some were continued until healthy growth ceased. In the twelve experiments 233 explants were cultivated. These and the controls were all fixed, usually in Zenker's fluid with 3 % acetic acid; they were embedded in paraffin and complete serial sections were cut at 8μ. Staining was with Mayer's haematoxylin, chromotrope and picric acid, or with Heidenhain's Azan.

Normal hair development was studied from the tenth day of embryonic life up to the adult condition. The normal skin fragments were fixed in Zenker's fluid with 3 or 5 % acetic acid, embedded in paraffin or in ester wax by the method of Steedman (1947), and cut and stained in the same manner as the cultures.

Living cultures and serial sections from both cultures and normal skin were examined with a polarizing microscope to observe the birefringence of hairs and other structures.

NORMAL DEVELOPMENT OF MOUSE HAIR FOLLICLES

Among the many good histological descriptions of the development of hair in mammals are one by Segall (1918) for the guinea-pig and one for the rat by Danneel (1931). Maurer (1892) figured two stages in the development of hair follicles in the mouse, and Oyama (1904) defined and illustrated four stages. In a study of pigmentation Hentschel (1930) figured the early and late stages of follicle differentiation found in the first few days after birth. Gibbs (1941) described the development of mouse hairs and follicles during the first 15 days after birth, and reported that the pre-natal development was similar to that in the Australian opossum, *Trichosurus vulpecula* (Gibbs, 1938). Dry (1926) studied the hairs of mice from birth to 4 months by dissection of the follicles.

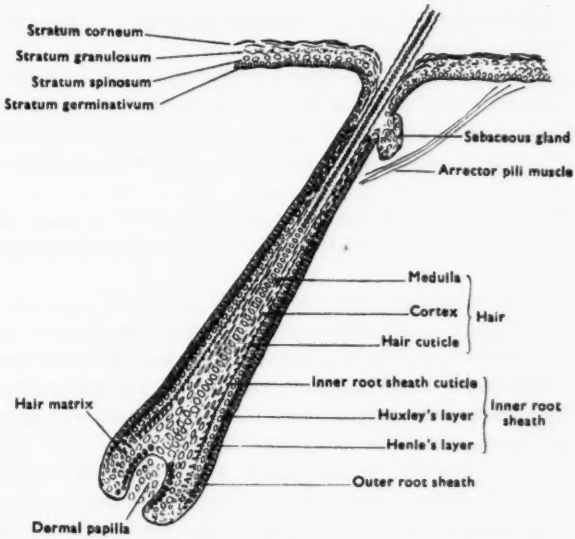
None of these authors correlated pre-natal hair follicle development in the mouse with changes in the skin or with the age of the embryos. A summary is therefore given of the main stages of development found in the present material. The summary is continued until the end of the first 4 weeks after birth and is correlated with the observations of Gibbs (1941) and Dry (1926). There was considerable variation in the length of the gestation period, but for this summary it is arbitrarily chosen as 21 days, and post-natal ages are recorded as the number of days from birth, prefixed by a 'plus' sign. The terminology for the mouse epidermis is that adopted by Hanson (1947). Reference is made to the measurements by Gibbs (1941) of the thickness of the epidermis and of the derma-plus-hypodermis. The names of the hair-follicle types are those proposed for mammals in general by Wildman & Carter (1939), but reference is also made to the terms used by Gibbs and by Dry. Since there are regional differences in the rate of hair development, the age recorded for each stage applies only to the lateral trunk region.

The adult mouse has no sudoriferous glands; the transitory sudoriferous gland rudiments reported by Gibbs (1941) were not seen. The main features of the structure of an active hair follicle are illustrated in Text-fig. 1.

Stage 0. *No follicles.* 12–13 days. The epidermis consists of stratum germinativum and periderm, sometimes also with a stratum intermedium one to two cells thick. No hair follicles are present. The derma is not differentiated from underlying meso-

derm. Blood vessels and nerves are present in the deeper part of the presumptive derma (Pl. 1, fig. 1).

Stage 1. *Follicle plugs.* 14 days. (Haarkeim stage of Oyama.) Plugs of epidermis extend into the mesoderm by the rapid division of cells of the stratum germinativum. There is a condensation of dermal cells at the base of each plug. These are the primary *X* follicles (primary follicles of Gibbs, monotrichs of Dry). The epidermis consists of stratum germinativum, stratum intermedium and periderm.



Text-fig. 1. Diagram of a fully developed mouse hair follicle.

Stage 2. *Pre-papillae.* 15 days. The bases of follicle plugs flatten prior to invagination. The epidermis consists of stratum germinativum, stratum spinosum and periderm. Cells of the panniculus carnosus muscle make their first appearance (Pl. 1, fig. 2).

Stage 3. *Papillae.* 16 days. (Haarzapfen stage of Oyama.) The bases of the follicle plugs are invaginated and rounded dermal papillae are formed. The epidermis has stratum germinativum, stratum spinosum, stratum granulosum and periderm (Pl. 1, fig. 4).

Stage 4. *Fibre cones.* 19 days. (Bulbuszapfen stage of Oyama.) The dermal papillae are longer than they are wide. In each follicle the elongated hardened cells of Henle's layer form a cone pointing toward the skin surface. The epidermis forms a stratum corneum (at 18 days).

Stage 5. *Hair canals.* 20 days. A space appears in the stratum spinosum and granulosum above each developing hair follicle. A slight swelling at one side of the follicle marks the position of the future sebaceous gland (Pl. 2, fig. 9).

Stage 6. *Hair formation.* 21 days, or at birth. (Beginning of Scheidenhaar stage of Oyama.) Inside the fibre cone of Henle's layer are distinguished the cells of

Huxley's layer, the inner root sheath cuticle and the tip of a hair fibre with a cuticle and cortex. Pigment is seen in the epidermal cells of the 'hair matrix' in some follicles. The epidermis is thick. Connective tissue fibres form a reticulum in the derma. Between the derma and the panniculus carnosus is a hypodermis of loosely arranged connective tissue with fat cells and blood vessels (Pl. 1, fig. 7; Pl. 2, fig. 9).

Stage 7. *Hairs in hair canals.* +1 day. The tips of the first hairs have reached the hair canals. Sebaceous cells appear in the sebaceous gland swellings. Primary *Y* follicles (secondary follicles of Gibbs, awls and auchenes of Dry) appear between the primary *X* follicles as epidermal plugs. The epidermis and the derma-plus-hypodermis are both increasing in thickness (Gibbs).

Stage 8. *Hairs emerged.* +2 to +3 days. The tips of the primary *X* follicles emerge from the skin. The primary *Y* follicles are differentiating (Gibbs). The epidermis and derma-plus-hypodermis are increasing in thickness (Gibbs). The periderm and outer layers of stratum corneum are shed while the hairs are emerging (Hanson).

The above stages refer to the complete differentiation of the primary *X* hair follicles, while stages 9–13 are concerned with the formation of primary *Y* and later follicles.

Stage 9. *'Trio' follicle groups.* +4 days. Most primary *X* and primary *Y* fibres have emerged. Primary *x* and primary *y* follicles (tertiary follicles of Gibbs, first underfur follicles of Dry) appear on either side of these to form groups of three follicles. The epidermis is decreasing in thickness and the derma-plus-hypodermis increasing (Gibbs). Connective tissue fibres in the derma form compartments around the trio groups. The hypodermis is now full of fat.

Stage 10. *Development of secondary follicles.* +5 to +8 days. Secondary follicles (quaternary follicles of Gibbs, further underfur follicles of Dry) are formed between the central and lateral members of the trio groups. From this time follicle groups are more difficult to distinguish. An arrector pili muscle becomes attached to each follicle (Gibbs). The epidermis continues to decrease in thickness and the derma-plus-hypodermis to increase (Gibbs).

Stage 11. *End of follicle length growth.* +14 days. All follicles cease to grow in length. The thickness of the epidermis remains constant, but that of the derma-plus-hypodermis is now diminishing.

Stage 12. *First resting phase.* About +18 days. The hairs have become thin at the base, their growth has ceased and 'hair bulbs' are being formed (the end of growth of G1 of Dry). The derma-plus-hypodermis is decreasing in thickness.

Stage 13. *Beginning of second growth cycle.* About +28 days. A new generation of 'companion hairs' (G2 of Dry) begins by proliferation of cells at the base of the resting hair follicles.

Melanin pigment is produced in the skin of mice and other mammals in the branched cells known as melanophores (Rawles, 1948) or pigmented dendritic cells (Billingham, 1948). Their colourless precursors, the melanoblasts, are derived from the neural crest (Rawles, 1940, 1947). They are to be distinguished from phagocytic cells in the derma which ingest but do not synthesize pigment granules, and which have not been found in normal mouse skin (Steiner-Wourlisch, 1925).

In the grey house-mouse Steiner-Wourlisch (1925) found melanophores first in

the derma of an 18 mm. embryo (i.e. 17–18 days' gestation, according to the data of Grüneberg, 1943). These pigmented cells became darker and more numerous after birth, and similar cells appeared in the basal part of the epidermis. Soon after birth the hair matrix contained melanophores. Hentschel (1930) found that as the hair grew, melanin granules appeared first in the cortex and then in the medulla.

In the C57 Black strain Rawles (1947) found melanophores were present at birth in the hair matrix and were lightly scattered in the derma. They were absent from the epidermis, except in sparsely haired body regions such as the ears and tail, where they were numerous in both the derma and the stratum germinativum.

THE DEVELOPMENT OF SKIN AND HAIR FOLLICLES *IN VITRO*

Skin pieces were cultivated for periods of from 1–3 weeks and most stages of differentiation of the epidermis, derma-plus-hypodermis and hair follicles were produced. The main results are summarized in Table 1, which shows the most advanced stage of differentiation reached in each experiment. In this section a summary of the results and of the effect of modifications of technique will be followed by a more detailed account of development *in vitro*.

(1) *The numbers of successful cultures*

The degree of success of the tissue-culture method may be judged to some extent from Table 1, but this refers only to the most advanced cultures in each experiment. Table 2 shows the number and percentage of cultures in which some new stages of follicle differentiation produced *in vitro* were recognized in histological sections. The percentages represent minimum values, since many cultures were fixed or discarded in the first few days, and in others progress was sometimes followed by retrogressive changes before fixation. At least 48% of all cultures were successful when all ages, body regions, techniques and growth media were included. When the incomplete figures for Exp. 9 are omitted the percentage is 54. Of the cultures from 12- to 14-day embryos, excluding Exp. 9, 68% were successful.

A further idea of the progress of cultures may be gained from the following details of Exp. 6, in which the percentage of cultures showing some differentiation was close to the average for the 12- to 14-day group:

26 cultures were prepared;
9 explants developed to Stage 6 (hair fibres);
6 explants developed to Stage 4 (fibre cones);
1 explant developed to Stage 3 (papillae);
2 explants developed to Stage 1 (follicle plugs);
7 explants grew without differentiation of hair follicles;
1 explant showed no outgrowth.

Two of the six cultures which developed to Stage 4 were fixed at 21 days, and might have produced hairs if cultivated for a longer period.

(2) *The effects of different conditions of cultivation*

(i) *Age of embryos.* From Table 1 it may be seen that the most advanced differentiation was obtained from embryos between 12 and 14 days old, but that younger and older tissues also differentiated to some extent. Table 2 shows that the percentage

of successful cultures was highest in the 12- to 14-day series of explants. The slight differences found sometimes between litter-mates in the stage of development were not large enough to affect the course of the experiments.

(ii) *Body regions.* Successful cultures were made with dorsal, lateral and ventral parts of the trunk. The mid-dorsal skin of 10- and 12-day embryos was not very suitable for explantation because the ectoderm was particularly thin and fragile,

Table 1. *Summary of tissue-culture experiments*

Exp. no.	Method	Age of embryo (days)	Initial stage	Final stage *in vitro*	Minimum duration of culture period for final stage reached (days)	Total age of explant at final stage (days)	Normal age *in vivo* for the stage reached *in vitro* (days)
11	Watch-glass	10	0	3	<19	<+8	16
12	Watch-glass	12	0	8	<18	<+9	+2 to +3
6	Hanging drop	12	0	8	13	+4	+2 to +3
9	Hanging drop	12	0	3	4	16	16
3	Hanging drop	12·5	0	5	8	20·5	20
10	Watch-glass	13	0	8	12	+4	+2 to +3
8	Hanging drop	14	0	5	9	+2	20
5	Hanging drop	14	1	8	10	+3	+2 to +3
1	Hanging drop	15	2	3–4	6	21	18
7	Hanging drop	15	2	6	7	+1	21
2	Hanging drop	18	3	6	3	21	21
4	Hanging drop	18	4	6	4	+1	21

Table 2. *Numbers of cultures which showed progress in hair follicle development*

Exp. no.	Age of embryo (days)	Total no. of cultures	No. of cultures showing further follicle differentiation	Successful cultures (%)
11	10	6	2	33
12	12	12	7	58
6	12	26	18	69
9	12	38	(8+)*	(21+)
3	12·5	10	8	80
10	13	11	11	100
8	14	36	18	50
5	14	14	12	86
1	15	20	4	20
7	15	36	15	42
2	18	14	6	43
4	18	10	4	40
	Grand total	233	113	48

* Not all the cultures were examined histologically to determine follicle differentiation.

and there was little underlying mesoderm. In normal development there are slight differences between regions in the rate of follicle differentiation, but equally great differences were found *in vitro* between explants from the same body region.

(iii) *Hanging-drop and watch-glass cultures.* It will be seen from Tables 1 and 2 that the two methods of cultivation were equally successful in producing follicle differentiation. The hanging-drop cultures contained only epidermis and mesoderm, so that the extra tissues such as cartilage included in watch-glass explants were evidently not essential for follicle development. In hanging-drop cultures the explants survived but did not show much progress after about 16 days *in vitro*, while explants in watch-glasses still showed growth of epidermis, derma and follicles after 25 days.

The longevity of the latter may be due to the greater original volume of the explants or to the provision of additional mesodermal tissue and the minimizing of its loss by migration. The introduction of other tissues sometimes resulted in a depression of hair-follicle development. Thus in some watch-glass cultures, when mammary gland rudiments were explanted with the ventral body wall, they grew vigorously at the expense of the follicles. This depression might be due to competition for nutrients, an accumulation of metabolites or a specific inhibitory effect. In Exp. 11 in which a variety of tissues was included in each explant from a 10-day embryo, the gut in ventral halves and the otic capsule in dorsal halves grew and differentiated very rapidly, but hair-follicle development was slow and limited to a few stages. The chief advantages of watch-glass cultures, then, were the provision of extra tissue for mechanical support, and the longevity of explants. The chief advantage of the hanging-drop method was the clear view of the living follicles.

(iv) *The position of explants in hanging-drop cultures.* In Exps. 1, 2 and 6 comparisons were made between two methods of placing explants in hanging-drop cultures. In the usual 'on plasma' method the explant was placed on the surface of the medium with the derma next to the medium. In the 'in plasma' method the explant was embedded in the clot with the epidermis next to the cover-slip In both cases the epidermis tended to roll up into a ball surrounded by derma, and there was no appreciable difference between the degree of differentiation of the cultures.

(v) *Size of explants.* In hanging-drop cultures the larger pieces of skin with sides of between 1 and 2 mm. were likely to produce more differentiation than smaller ones.

(vi) *Incubation temperatures.* The effects on growth of temperatures of 34 and 37°C. were compared in Exps. 1, 2 and 6. The general health of the cultures was very similar, and there was no appreciable difference between the rates of hair-follicle development.

(vii) *Frequency of subcultivation.* In both hanging-drop and watch-glass cultures the explants were transferred to fresh medium at intervals varying from 3 to 6 days. Intervals of 3 or 4 days were usually not too great for the health of the tissues, and older cultures could be left for 5 or 6 days. Explants incubated at 34°C. did not need subculture quite so often as those incubated at 37°C.

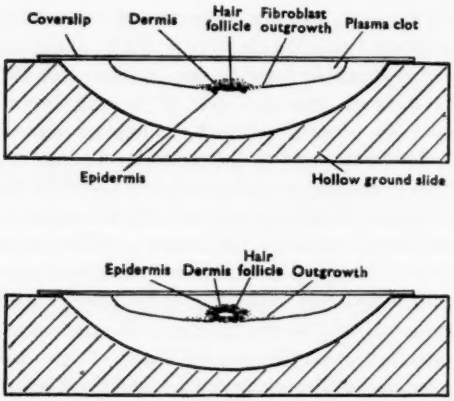
(viii) *Saline component of the medium.* In the first ten experiments Tyrode solution was used for washing explants and for preparing the chicken-embryo extract. In Exps. 11 and 12 a comparison was made between Tyrode and Pannett and Compton saline, which lacks glucose and has a different salt balance. No differences were observed between the two groups of explants.

(ix) *Duration of experiments.* Each experiment had a slightly different purpose and was therefore continued for a different period. Hanging-drop cultures were maintained for from 7 to 17 days, and usually ceased to grow after about 16 days. Some of the watch-glass explants were still growing when fixed after from 18 to 25 days *in vitro*.

(3) *The general appearance and orientation of explants*

Undifferentiated cells migrated into the medium from all healthy explants. The outgrowth was more extensive in hanging-drop than in watch-glass cultures. Fibroblasts predominated, but epithelial cells were also found in the outgrowth from the skin of older embryos and from all explants after two or three passages.

Skin pieces from 12- to 14-day embryos, which were spread flat on the surface of a plasma clot in hanging-drop cultures, nearly always curled up at the edges on the epidermal side (Text-fig. 2). This was associated with a contraction of the ectoderm and with the greater rate of proliferation of mesoderm around and beneath it. After a few days the epidermis frequently rolled into a hollow sphere surrounded by mesoderm. Hair-follicle plugs extended into the mesoderm in directions roughly normal to the dermal-epidermal junction, so that in a flat explant they were more or less parallel to one another but in a spherical one they radiated from the central epidermis in all directions. In the latter case the tips of the hair fibres then grew towards the



Text-fig. 2. The orientation of skin explants in hanging-drop cultures. Cross-sections through the centre of culture chambers are represented. In the upper diagram the skin is on the surface of the clot and the epidermis is beginning to curl at the edges. In the lower diagram the epidermis of an older culture has rolled into a ball.

centre of the explant. A great practical advantage of this orientation was that the development of follicles could be clearly seen around the edges of the explants without vision being obscured by the thickening epidermis.

In most of the hanging-drop cultures from 15- or 18-day embryos the explants curled in the opposite direction, so that the epidermis surrounded the derma. The hair follicles were then imprisoned in a sphere of keratinizing epithelium, and showed the effect of isolation from the nutrient medium by their small size and delayed development.

In the watch-glass cultures, in which the explants were many times larger than in hanging-drop preparations, and which contained cartilage and a greater quantity of mesoderm, the epidermis again tended to roll into a ball surrounded by mesoderm. This feature was noted also in rat and mouse embryonic skin in tissue cultures by Hanson (1949), in mouse skin on the chick chorio-allantois by Reed & Alley (1939) and in mouse skin on the chick coelom by Rawles (1940, 1947).

(4) *The histogenesis of the epidermis*

Hanson (1949) studied the differentiation *in vitro* of epidermis from 12- and 16-day rat embryos and 16-day mouse embryos. From the earliest stage, a single layer of cells in the case of the 12-day rats, a fully differentiated epidermis of stratum germinativum, stratum spinosum, stratum granulosum and stratum corneum was produced in culture. This differed from normal epidermal histogenesis only in the persistence of the periderm and the formation of a hollow cyst by curling of the epidermis. The present observations fully confirmed those of Hanson on the 16-day mouse embryo, and extended the investigation to 10- to 15-day explants. The results may be seen by examining Figs. 1–10, which show that the rate of differentiation was comparable with that *in vivo*. The periderm and all layers of the stratum corneum persisted in culture and eventually caused distension of the epidermal cyst (Pl. 2, fig. 11). Where the stratum corneum was on the outside of the culture and in contact with the plasma, it was frequently abnormal in appearance.

(5) *The partial histogenesis of the derma*

The mesodermal cells of explants made at Stage 0 multiplied rapidly and many migrated outwards as fibroblasts (Pl. 1, figs. 3, 5). In hanging-drop cultures much presumptive dermal tissue was lost by outward migration, and even in watch-glass cultures where this tendency was minimized, the derma of old cultures was thinner than in the young mouse. By 21 days, as in normal development, a reticulum of connective tissue fibres had formed in the derma (Pl. 1, fig. 8; Pl. 2, fig. 10). These fibres were predominantly parallel to the dermal-epidermal junction. There was no suggestion of a regular fatty layer corresponding to the hypodermis of normal development.

Smooth muscle fibres were sometimes seen, but these were not regularly attached to hair follicles as arrector pili muscles. When parts of the panniculus carnosus were included in cultures, they sometimes persisted.

The immature blood cells in vessels of the mesoderm survived for some days but eventually degenerated. Nerve fibres were not seen in cultures, and presumably they, too, degenerated.

(6) *The histogenesis of hair follicles*

The first eight stages of hair-follicle development described above, from the appearance of epidermal plugs to the emergence of hairs from the skin, were produced *in vitro*; thus the complete histogenesis of an individual hair follicle may be accomplished in a single tissue culture. Even skin from 12-day embryos, 2 days before normal follicle development (Pl. 1, fig. 1), was capable of producing hairs in culture, skin from 13- and 14-day embryos was equally successful, and follicle development began in cultures from 10-day embryos (Table 1). Stages 9–13, which were not reported *in vitro*, refer only to the development of new follicles and to the cessation of hair growth.

The general shape of the hair follicles and the detailed structure of the dermal papilla, root sheaths and growing hair were strikingly similar to those in normal embryos and young mice. This is best seen by a study of Figs. 2–10.

There was much variation between individual explants, but those with healthy growing derma and epidermis usually had a number of normal hair follicles. In general, hair-follicle development *in vitro* in the most successful cultures corresponded exactly to that *in vivo* up to the stage of the 2-day old mouse in which the tips of the hairs are emerging (Stage 8). After that, however, there was little further change in the explants, although the formation of hair from the existing follicles and of stratum corneum continued for some time. Instead of becoming thick, the hairs remained very fine and did not develop a regular medulla; their growth in length was less rapid than in the young mouse, and there were no well-defined clusters of new follicles around the older ones. The longest follicles, lacking the support of a fatty hypodermis, usually lost their radial arrangement and tended to lie parallel to the skin surface (Pl. 2, fig. 11). Sometimes the epidermal plugs of new follicles which developed later than the reticular fibres of the derma seemed unable to push through this layer, and for that reason they too remained near the epidermis. The sebaceous glands arising from epidermal swellings at the side of the follicles (Pl. 1, fig. 8) sometimes produced a few differentiated sebaceous cells (Pl. 3, fig. 18), but did not enlarge further.

(7) *The rate of hair-follicle development*

In Table 1 a comparison is made between the time taken *in vitro* to reach the most advanced stage of follicle differentiation in each experiment, and the time taken *in vivo* to reach this stage on the lateral trunk region. In some cases the development times recorded for cultures were too long because the explants were only fixed at intervals of 3 or 4 days. However, the table indicates that in most experiments differentiation in the best cultures was about as rapid as it is in normal development. The conclusion was the same when intermediate stages were examined. For example, Stage 5 (20 days *in vivo*) was reached by cultures at 20 days in Exp. 12 (8 days *in vitro* from 12-day explants) and at 21 days in Exp. 10 (13 days *in vivo* and 8 days *in vitro*). Stage 6 (21 days *in vivo*) was reached at 21 days in Exp. 6 (12 days *in vivo* and 9 days *in vitro*) (Pl. 1, fig. 8) and at +1 day in Exp. 5 (14 days *in vivo* and 8 days *in vitro*). Figs. 2–10 illustrate the similarity of rates of development *in vivo* and *in vitro*.

In hanging-drop cultures it was often possible to observe daily the stage of follicle development, checking the final stage by histological examination. The rates of development of a few typical cultures are compared with the normal rate in Table 3. It will be seen that follicle development in cultures was at about the same rate as in the embryo, although sometimes a day or two faster or slower. On the whole, agreement was remarkably good. There were, of course, some less healthy cultures in which development was slower, and some in which differentiation was followed by retrogressive changes.

(8) *Mitotic activity*

There is a high mitotic rate in the normal skin of embryos and young mice. Hanson (1947) found mitoses in from 2 to 4% of all nucleated epidermal cells between 10 and +10 days. These occurred only in the stratum germinativum and for a short period in the periderm and stratum spinosum.

Qualitative examination of the present material showed that the mitotic rate is high in the stratum germinativum between follicles and still higher in those parts which are forming follicle plugs. Cells of the outer root sheath continue to divide for at least 10 days after birth, while the follicles are increasing in length. Those cells which form the hair matrix divide actively during the period of hair growth. Mitoses are also frequent in the derma for some time after birth.

Table 3. *Stages of follicle development in some living cultures*

Age in days	Stage *in vivo*	Exp. 5 (age of embryo, 14 days)		Exp. 6 (age of embryo, 12 days)			
		No. 61	No. 63	No. 105	No. 108	No. 112	No. 115
13	0	—	—	0	1	1	0
14	1	1	1	0	1	1	0
15	2	1	1	1	—	1	1
16	3	2	2	1–3	3	2	—
17	3	3	2–3	3	3	2	3
18	3	3	3	3	3	3	3
19	4	3–6	3–4	4	4	4	4
20	5	3–6	4	4	4–6	4–6	4–6
21	6	6	6	4–6	6	6*	4–6
+1	7	7	7	4–6	6–8	—	6
+2	8	8*	7–8	6	6–8	—	6
+3	8	—	8	6	6–8	—	6
+4	9	—	8	6*	8*	—	6*

* From histological examination.

In general, healthy explants resembled normal skin in the distribution and frequency of mitoses during follicle development. Mitoses were present for a fairly long time *in vitro*. Even after 25 days' cultivation (no. 246 in Exp. 10), dividing cells were still found in the epidermis, derma and hair follicles.

(9) *The size of follicles and hairs*

In the embryonic period the healthiest explants developed follicles not only at the same rate as *in vivo* but also of the same size. In the early stages the follicles were sometimes even larger than their counterparts in the embryo, as may be seen by comparing Fig. 3 with Fig. 2 at twice the magnification. In cultures in which the general health was poor, the follicles were often abnormally small. This was well illustrated in a few explants such as no. 249 in Exp. 10, in which follicles at Stage 3 situated near the culture medium were very large, while others at the same stage but separated from the medium by a fold of epidermis were abnormally small.

Just after birth, the fully keratinized hairs in the largest follicles of normal skin are from 3 to 6μ in diameter, and the diameter of follicles at the level of keratinized hairs is about ten times greater. Some follicles are about six times longer than they are wide. The fibres are circular in cross-section and consist of a cortex surrounded by annulate cuticular scales, as described by Dry (1926). Many follicles and fibres of similar shape and size were produced *in vitro* (Pl. 2, figs. 11–15).

In the first fortnight after birth the follicles in normal skin double or treble their length and the hairs become much thicker, although there is great variation between follicle types, body regions, individuals and strains in these characters. Primary *X*

and *Y* fibres may become as much as 30μ wide for a short distance, and diameters of from 10 to 14μ are frequent in all fibre types. Nearly all fibres develop a septate or septulate medulla (Dry, 1926). The medulla occupies half or more of the width of the fibres, so that the increase in volume of keratinized cortex is not as great as fibre thickness measurements would suggest. Some hairs reach a length of 6 mm. or more. At about +9 days primary *Y* follicles and fibres become kidney-shaped in cross-section (Gibbs, 1941).

By contrast, the hairs and follicles in cultures remained small. Follicles sometimes elongated after Stage 8, but not to the same extent as in normal skin. Fibre thickness reached from 6μ (Pl. 2, fig. 14) to 11μ (Pl. 2, fig. 11), but rarely more. Occasionally there were traces of medullation, but no regularly septate medullae were formed. Most fibres were round or oval in cross-section and none were kidney-shaped. Growth in length was much slower and no fibres longer than 0·5–1·0 mm. were found.

(10) *Follicle types and follicle population density*

The hair follicles which appeared first in culture may be identified with the primary *X* follicles of the embryo. In normal development the earliest of these follicles are widely spaced at approximately equal distances from one another, and new follicles then appear in the spaces between them (Pl. 1, fig. 4). The same tendency was observed in some cultures (Pl. 1, fig. 5). At Stage 7 *in vivo* the primary *Y* follicles appear, forming irregular rows with the primary *X* follicles (Gibbs, 1941). In cultures new follicles appeared at about the same stage, but they were not arranged in rows. The later distribution of follicles in groups which is characteristic of mammalian skin and which is definite, though transitory, in the mouse (Gibbs, 1941), was not observed *in vitro*.

Normal follicle grouping and density could hardly be expected in cultures where the skin was no longer stretched over an expanding body surface but was curled up in a ball. No counts were made of the number of follicles per unit of skin area, but in the younger cultures at least the density was roughly comparable with that of corresponding stages *in vivo* (Pl. 1, figs. 2–8).

(11) *The fine structure of hairs*

Each hair grown in tissue culture consisted of a cortex surrounded by cuticular scales (Pl. 2, figs. 11–15). Both layers were formed from epidermal cells of the hair matrix by cytological changes similar to those observed in normal skin. Hair formation in culture was preceded by the appearance of an outer root sheath, Henle's layer, Huxley's layer and the cuticle of the inner root sheath in exactly the same manner as *in vivo* (Pl. 2, fig. 13). The 'keratinized' fibre in the upper part of the follicle and above the skin surface stained differentially in saturated aqueous picric acid in the same manner as the 'keratinized' part of normal hairs, while the hair in deeper parts of the follicle remained unstained with picric acid both *in vivo* and *in vitro*. The physico-chemical basis for the very specific retention of picric acid by the hairs when it is lost from the stratum corneum, root sheaths, medulla and all other skin structures is unknown, but it is a useful distinguishing feature of hair in normal histology. This 'keratinized' portion of hairs fails to take up a wide variety of histological stains in both normal and cultivated skin.

The birefringence of mouse hair follicles was similar to that of human hair which has been described by Schmidt (1934). Freshly plucked mouse hairs mounted in water show an intense positive uniaxial birefringence of the cortex in the direction of the shaft. A weak birefringence is observed a short distance above the papilla, and this increases to maximum intensity near the zone of maximum staining with picric acid. The examination of sections shows that birefringence in the 'keratinized' part of the hair is very much stronger than that of the same thickness of hair in the deeper part of the follicle or than that of the stratum corneum or inner root sheaths. Parts of the inner root sheath are birefringent in the direction of the hair shaft, while the stratum corneum shows orientation in the plane of the skin surface.

In sections of tissue cultures the inner root sheaths, hairs and stratum corneum had normal birefringent properties. The hairs were weakly birefringent in the deeper parts of the follicles, but in the 'keratinized' portion they were very much brighter than any other structures in polarized light. Hairs in living cultures were also strongly birefringent.

The observations described above show that there is a close similarity between the histogenesis of hairs *in vitro* and *in vivo*. Histochemical studies of hair development in the normal animal and in tissue cultures will be reported elsewhere.

(12) *Pigmentation*

Melanin pigment was produced *in vitro* in the two experiments with watch-glass cultures from pigmented strains of mice. No pigment cells were found in the watch-glass or hanging-drop cultures from the embryos of albino mice.

In Exp. 10, 13-day litter mates of the C57 Black strain were used. Each explant consisted of half of the ventral body wall, with epidermis, mesoderm and developing cartilage. Branched melanophores were found in all of the six explants fixed at +4 days or later (i.e. after 12 or more days *in vitro*), and in one of the two fixed at 21 days (after 8 days *in vitro*). These cells could be distinguished in the living cultures, appearing first in the derma (Pl. 3, fig. 16) or in the derma and hair follicle matrix (Pl. 3, figs. 19, 21) between 21 and +2 days. Pigmented hairs (Pl. 3, figs. 20–22) were first recognized between +6 and +8 days, and sections revealed that melanophores were sometimes present also in the deeper part of the epidermis. In one explant, in addition to melanophores in the stratum germinativum, pigment granules were seen in some cells of the stratum spinosum (Pl. 3, fig. 17).

In Exp. 12 the source of the 12-day embryos was the laboratory stock of mice of agouti colouring. No melanophores were found in any of the five explants of dorsal or ventral body wall fixed at 21 days. They were present, however, in two of the three explants of dorsal body wall fixed at +5 days and in one of two explants of ventral body wall fixed at +9 days. It was seen from the living cultures that melanophores appeared in these explants between 18 and +2 days, and pigmented hairs between +5 and +7 days. The melanophores were first seen in the derma or in both the derma and hair matrix. Sometimes they were recognized early as pale branched cells which became gradually darkened with melanin in the next 1 or 2 days. The histological sections showed melanophores in the epidermis as well as in the derma and hair matrix. Pigment granules were found in the cortex of hairs in two explants, and in one of these some of the differentiating cells of the epidermis also contained

pigment. Hair-follicle development was a little slower in these cultures than *in vivo* (Table 1), and when this is taken into account the time of appearance of melanophores in various layers of the skin can be regarded as normal.

Another interesting observation was made in certain cultures in each of the above experiments. Some of the hair follicles failed to produce normal keratinized fibres *in vitro*, forming only cords of nucleated cortical cells. Many such follicles had melanophores in the hair matrix, and pigment was produced and extruded as though normal hairs were present to receive it, so that a cylinder of clumped melanin granules was pushed to the skin surface (Pl. 3, figs. 18, 19). It was not possible to determine whether the pigment cylinders consisted of dead melanophores or merely of granules deposited from them. The process was observed both in living explants and in fixed material. The absence of normal hair formation was confirmed in sections, where there was no normal hair outline, marked picric acid affinity or strong birefringence in the pigment cylinders. Sometimes normally keratinized and pigmented hairs were found in the same explant as these pigment masses (Pl. 3, fig. 22). A few irregular pigment masses were found extracellularly in the derma in some old cultures (Pl. 3, fig. 16), but these were much less conspicuous than the cylinders from hair follicles. When an explant contained many hair follicles the melanophores were concentrated in them, but when hair-follicle formation was deficient, the pigment cells were numerous in the derma and sometimes also in the epidermis. No extensive migration of individual melanophores was observed and none was found in the outgrowth of fibroblasts and epithelial cells.

DISCUSSION

Problems of histogenesis

Nothing is known of the factors which induce hair-follicle formation from the mammalian skin. The cause of the growth of new hair follicles from the base of old ones in the hair cycles of rodents is also unknown, although this is associated with an increased blood supply (Haddow, Elson, Roe, Rudall & Timmis, 1945), and with many other physiological factors. It is interesting to note, therefore, that in the present experiments hair follicles formed *in vitro* in the absence of any tissues except the epidermis and underlying mesoderm. Chemo-differentiation of the skin for hair-follicle formation apparently occurs before the tenth day in the mouse embryo, and the work of Rawles (1947) with coelomic grafts suggests that it may take place as early as the eighth day. Tissue cultures have shown also that neither a blood circulation nor nervous stimulation is necessary for the initiation of follicles, although both blood vessels and nerves were present in the mesoderm at the time of explantation. Any influence of fluids normally bathing the surface of the embryo is also excluded. A factor stimulating the formation of hair follicles is extremely unlikely to be present in a culture medium of avian origin, especially as the time of follicle initiation depended on the total age of the tissues and not on the duration of cultivation. It seems most probable that the embryonic ectoderm and mesoderm are not dependent on any outside influences for follicle formation.

The pattern of hairs in the skin presents many interesting problems. As in other mammals, the first, or primary *X* follicles in the mouse are rather regularly spaced,

and later follicles appear in positions related to them. Either this pattern is already laid down in the skin, or each developing follicle becomes surrounded by a small zone where development is inhibited. There are difficulties in accepting either theory. The present studies have thrown little light on this problem except to show that an equally regular spacing of primary *X* follicles can be obtained in skin removed from blood capillaries and nerves.

Not only could the differentiation of hair follicles begin *in vitro*, but it could proceed to completion with all the many morphologically distinct layers in normal relation to each other. This complete self-differentiation took place in a relatively constant medium, entirely avian in origin. The follicle parts were formed in the right order and at the normal rate, and they were the same size as those *in vivo* during the differentiation period. Thus the medium, at least qualitatively, lacked none of the essential requirements for follicle differentiation and hair growth from ectoderm and mesoderm.

In the fowl it was shown by Lillie & Wang (1944) that the dermal component of a feather papilla induces differentiation in the epidermal component. It is possible that a similar state of affairs exists in the hair follicle; this has not been investigated. In tissue cultures it was noted that no epidermal plugs formed in the absence of mesoderm, and that dermal papillae could not be distinguished in areas from which the epidermis had been removed.

Hair canal formation

Gibbs (1938) described the events leading to the formation of a hair canal in the epidermis above a developing follicle in *Trichosurus vulpecula*. A plug of cells connected with the differentiated sebaceous gland pushes outwards, causing the cells of the stratum spinosum in front of it to disintegrate, forming a small canal which is quickly keratinized. A similar process was reported in the sheep (Wildman, 1932) and the mouse (Gibbs, 1941), but not in mammals generally, where the formation of the hair canal is usually regarded as a function merely of the upper layers of the epidermis.

In the present study, a connexion between the sebaceous gland and the formation of a hair canal was not satisfactorily established in normal mouse skin.

Hair canals in the mouse were observed *in vitro* in hanging-drop cultures, both in the living tissue and in sections. Although in explants a sebaceous gland swelling usually appeared at the time of hair canal formation, no differentiated sebaceous cells were seen at this period. The few follicles which produced differentiated sebaceous cells did so at a much later stage. The hair canals were formed by the keratinization of cells in the epidermis, and appeared to be quite normal. No connexion was observed between the sebaceous gland swelling and the hair canal, and it seems that in the mouse skin, at least *in vitro*, differentiation of the gland cells is not essential for the development of a hair canal.

Pigmentation

Rawles (1940, 1947), using grafts to the chick coelom, showed that the precursors of pigment cells had migrated to all parts of the skin of the mouse by the twelfth day of gestation. The present observations on tissues cultivated in a non-cellular

medium confirm this result. Unlike those of dominant and recessive white breeds of fowls (Hamilton, 1940) tissues from the albino mouse did not produce melanophores *in vitro*. However, it is possible that white dendritic cells are present, as in spotted guinea-pigs and albino rabbits (Billingham, 1948) or that the melanophores are less viable than in the pigmented strains, as is the case with fowls (Hamilton, 1940).

The differentiation *in vitro* of mammalian melanophores is apparently very similar to the process in tissue cultures from birds (Koller, 1929; Dorris, 1938; Hamilton, 1940). To the knowledge of the present writer the only previous observation of mammalian pigment cell differentiation in culture was that of Murray (1933*a*). The deposition of pigment granules from melanophores into the medium was observed in chick explants by both Dorris and Hamilton, while Grand & Cameron (1948) described the same process in cultures of mouse melanoma tissue. It has now been demonstrated that melanophores can also deposit their pigment in the hair cortex *in vitro*. The process was similar to that in the normal mouse as described by Steiner-Wourlisch (1925). The few differentiating epidermal cells which contained pigment were first seen after the epidermal melanophores had appeared and were only found in areas where the latter were numerous. It therefore seems likely that melanin in these epidermal cells also was derived from melanophores.

No extensive migration of dendritic cells was observed after they had become pigmented, and in this respect they resembled the fowl melanophores *in vitro* (Dorris, 1938; Hamilton, 1940).

No evidence was found in support of the belief of Steiner-Wourlisch (1925) and Hentschel (1930) that the epidermal and hair-matrix melanophores are independent of the dermal melanophores. On the contrary, observations *in vitro* support the suggestion of Rawles (1947) that there is a balanced distribution of melanophores between the three regions. No morphological difference was observed *in vitro* between pigment cells of the derma and epidermis.

Although the pigmentation process in normal skin and hair is easily upset by changes in the environment (Reed & Henderson, 1940; Butcher, 1945), melanophores of the mouse skin in tissue culture were much less sensitive than those of pigmented breeds of fowl (Hamilton, 1940). Pigmentation could even occur normally *in vitro* in hair follicles in which normal keratinization was not taking place. The tissue-culture technique may, therefore, be useful for studying some of the outstanding problems of melanin formation in mammals.

Comparison of the tissue-culture technique for studying hair growth with some other experimental methods

The present experiments on embryonic mouse skin were much more successful in producing the differentiation *in vitro* of the follicles of pelage hairs than were those of Strangeways (1931) on the guinea-pig or Murray (1933*b*) on the rat. Strangeways cultivated the skin of embryos of various ages but apparently did not obtain hair-follicle differentiation of more than one or two stages. Both she and Murray (1933*b*) referred to the thick stratified epidermis which interfered with the normal development of the follicles of pelage hairs. It may be that the thinner epidermis of the mouse is more suitable for *in vitro* studies of hair follicles.

Varying degrees of success in hair-follicle development were reported by those using the chick chorio-allantoic graft method (Hiraiwa, 1927; Nicholas & Rudnick, 1933; Reed & Alley, 1939). Even the most successful and highly vascularized grafts of Reed & Alley did not persist as long or produce follicle differentiation as consistently as the tissue cultures.

Grafts of the embryonic skin of the mouse to the chick coelom were much more satisfactory. In the first experiments by Rawles (1940) 61% of grafts 'took', and in a later series (Rawles, 1947) 77% were successful. Hair follicles and hairs were produced in most of these implants. Rawles noted that hair development was more abundant from 11- to 12-day embryonic grafts than from 8- to 10-day tissues. An illustration to the second paper shows that one graft at least had medullated hairs from 7 to 12μ thick and about 700μ long. At a total age of +10 days, some hairs were said to be several millimetres long. The hair follicles were normal in histological detail and very similar to those grown in tissue cultures in the present experiments. Tissue cultures and coelomic grafts thus illustrate to a similar degree the capacity of mouse skin and hair follicles to differentiate in an avian environment. A greater volume of keratinized hair was produced by the coelomic graft method, probably because of the presence of a blood circulation.

It is considered that the tissue-culture techniques described in this paper possess some advantages over all of the grafting techniques for the purpose of studying the effects of the non-cellular environment. The nutritive medium, though complex, is relatively constant, and its chemical constituents may be analysed at any time during the experiment. Any nutritive substance, enzyme or inhibitor may be added to the medium to produce its effect directly on the skin. Tissue culture permits the continuous observation of histological changes in hair follicles in a way that is not possible by any other technique.

Limiting factors to continued hair growth

There are many possible reasons why hair growth cannot at present be maintained indefinitely. Since differentiation and the synthesis of hair keratin is completed in culture, nutritional deficiencies are likely to be quantitative rather than qualitative. There may be too little of the raw material or of the enzymes for hair synthesis, or these may be in the wrong proportions. It is interesting to note that there was no apparent reduction in the rate of formation of keratinized stratum corneum. The stimulus to cell division may become inadequate, yet a high mitotic rate is maintained in the epidermis. Mechanical factors arising from the abnormal orientation of follicles may be partly responsible, but they did not prevent good hair growth in the chick coelom grafts of Rawles. The better results from coelom grafts suggest the importance of vascularization.

Some of these possibilities will be investigated in future work.

SUMMARY

1. Skin from the trunk of mouse embryos of 10–18 days' gestation was grown in hanging-drop and watch-glass cultures on a mixture of fowl plasma and chicken-embryo extract.

2. Some differentiation of hair follicles took place in embryonic skin of all ages, but the best results were obtained from the skin of 12- to 14-day embryos.

3. All stages of hair-follicle development up to the emergence of hairs from the skin were produced from undifferentiated ectoderm with some underlying mesoderm.

4. The histological structure of the hair follicles and hairs formed in culture was normal.

5. In the most successful cultures, follicle differentiation took place as rapidly as in the embryo, and the follicles and hairs were of normal size. When the tissues reached an age corresponding to the second day after birth, hairs continued to grow for about a week but they did not become as large as in the young mouse.

6. Dendritic cells appeared in culture, developed melanin granules and deposited these in growing hairs *in vitro*.

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EXPLANATION OF PLATES

Photographs by Mr V. C. Norfield and Mr G. Lenney.

C	Nucleus of hair cortex cell	*MG*	Melanin granule
Cr	Stratum corneum	*Ms*	Mesoderm
Cu	Nucleus of hair cuticle cell	*O*	Outer root sheath
CuI	Nucleus of inner root sheath cuticle	*P*	Dermal papilla of hair follicle
F	Fibre cone	*PC*	Panniculus carnosus muscle
G	Stratum germinativum	*PP*	Pre-papilla
Gr	Stratum granulosum	*Pr*	Periderm
H	Hair	*R*	Reticular layer of derma
HC	Hair canal	*S*	Stratum spinosum
He	Henle's layer	*SG*	Sebaceous gland swelling
Hx	Huxley's layer	X_1	Early primary *X* follicle
M	Melanophore	X_2	Later primary *X* follicle

PLATE 1

Fig. 1. Vertical section of skin of 12-day mouse embryo at Stage 0, showing the epidermis and underlying mesoderm with immature blood cells (control to Exp. 6). Haematoxylin, chromotrope. ×572.

Fig. 2. Vertical section of skin of 15-day mouse embryo at Stage 2, with two early follicle plugs and one larger follicle at the pre-papilla stage (control to Exp. 7). Haematoxylin, chromotrope. ×185.

Fig. 3. Horizontal section of an entire culture of skin from a 12·5-day mouse embryo after 3 days *in vitro* (no. 37, Exp. 3). The explant has reached Stage 2, and several hair follicles are seen in the section. Haematoxylin, chromotrope. ×93.

Fig. 4. Horizontal section of skin of a 16-day mouse embryo at Stage 3, showing the pattern of early and late primary *X* follicles. Haematoxylin, chromotrope. ×59.

Fig. 5. Horizontal section of an entire culture of skin from a 14-day mouse embryo after 3 days *in vitro*, showing follicles at Stage 3 (no. 66, Exp. 5). Haematoxylin, chromotrope. ×59.

Fig. 6. A deeper section of the same culture showing the arrangement of the earliest primary *X* follicles in rows. Haematoxylin, chromotrope. ×59.

Fig. 7. Oblique section of skin of newborn mouse at Stage 6, showing hair follicles of various sizes, the largest containing minute hair tips inside the fibre cones. Haematoxylin, chromotrope, picric acid. ×90.

Fig. 8. Horizontal section of a culture of skin from a 12-day mouse embryo after 9 days *in vitro*, at Stage 6 and showing many hair follicles (no. 129, Exp. 6). Haematoxylin, chromotrope, picric acid. ×90.

PLATE 2

Fig. 9. A hair follicle in the skin of a newborn mouse at Stage 5. The epidermis is fully differentiated, and two younger follicle plugs are also seen. Azan. ×243.

Fig. 10. Part of a culture of skin from a 12·5-day mouse embryo after 8 days *in vitro*, showing a follicle at Stage 5 (no. 45, Exp. 3). The epidermis is fully differentiated and the centre of the explant is filled with stratum corneum. Azan. ×243.

Fig. 11. Portion of a culture of skin from a 14-day mouse embryo after 16 days *in vitro*, showing two follicles lying in the derma parallel to the skin surface (no. 63, Exp. 5). Haematoxylin, chromotrope, picric acid. ×270.

Fig. 12*a*, *b*, *c*. Three adjacent sections of a hair follicle in skin from a 13-day mouse embryo after 12 days *in vitro* (no. 250, Exp. 10). Haematoxylin, chromotrope. ×369.

Fig. 13. Part of the section shown in fig. 12*b* at higher magnification showing the follicle sheaths and the formation of the hair from the cortex and cuticle cells. Haematoxylin, chromotrope. ×1260.

Fig. 14. The field shown in fig. 13 at a different focal depth to show the surface of the hair with its cuticular scale pattern. Haematoxylin, chromotrope. ×1260.

Fig. 15. A hair emerging from its follicle mouth in a culture from a 13-day mouse embryo after 19 days *in vitro* (no. 241, Exp. 10). Haematoxylin, chromotrope, picric acid. ×225.

PLATE 3

Fig. 16. Part of a culture from a 13-day mouse embryo after 25 days *in vitro*, showing two melanophores in the derma (no. 246, Exp. 10). Haematoxylin, chromotrope, picric acid. ×765.

Fig. 17. Part of the epidermis in the same culture, showing melanophores in the stratum germinativum and processes from these cells between the cells of the stratum spinosum. Some epidermal cells in the stratum spinosum and stratum granulosum contain melanin granules. Haematoxylin, chromotrope, picric acid. ×396.

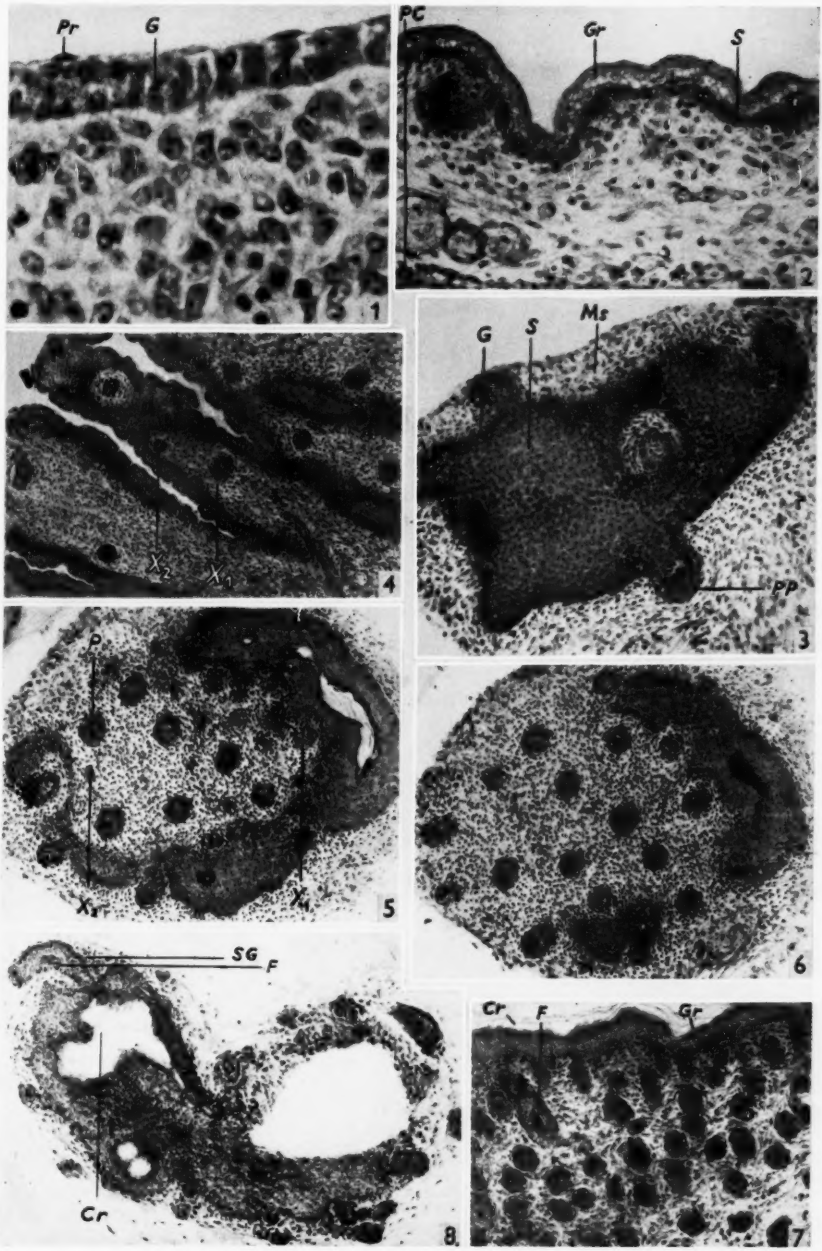
Fig. 18. Section through a hair follicle in the same culture, showing melanin granules in the follicle above the dermal papilla, and a small sebaceous gland. Haematoxylin, chromotrope, picric acid. ×603.

Fig. 19. Part of a culture from a 13-day mouse embryo after 22 days *in vitro*, showing a cylinder of melanin pigment which has been extruded from a follicle (no. 247, Exp. 10). Haematoxylin, chromotrope, picric acid. ×599.

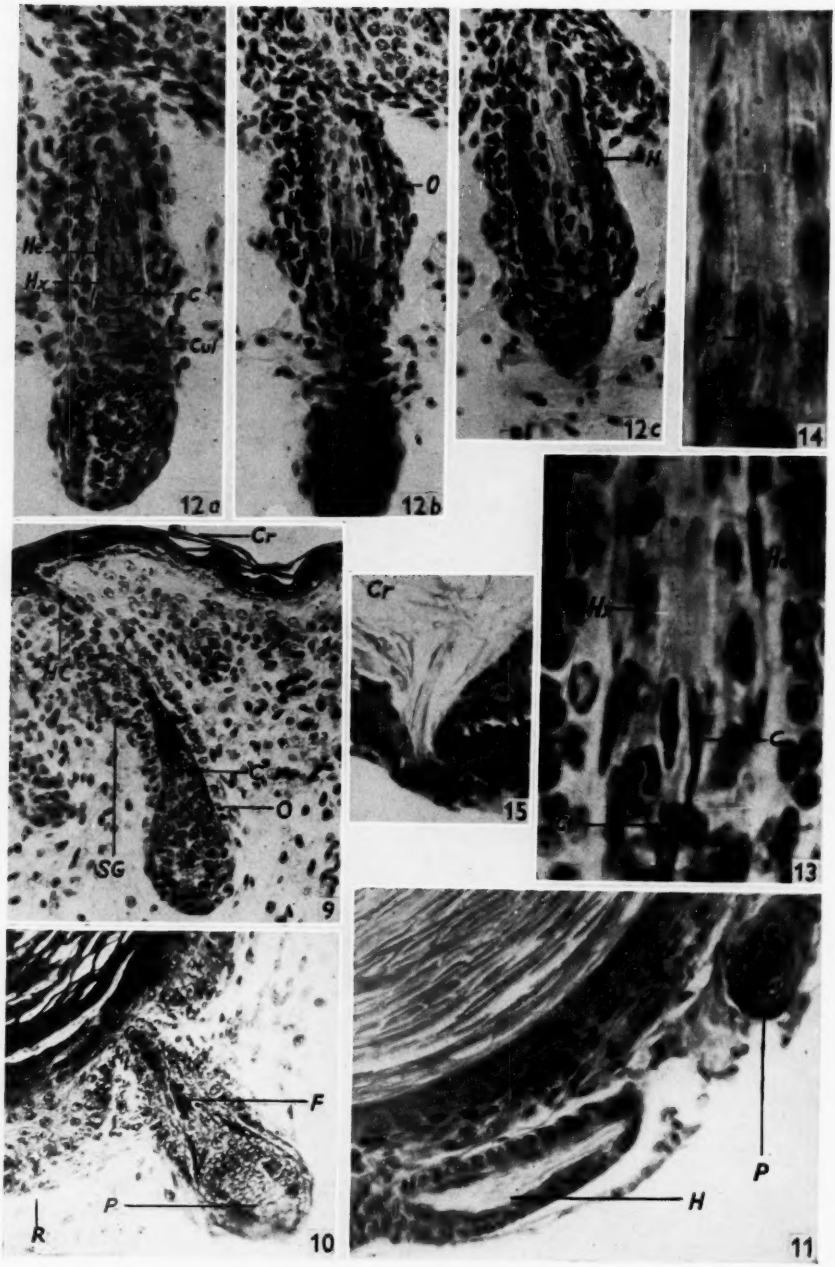
Fig. 20. Part of the same culture, showing a normal pigmented hair. Haematoxylin, chromotrope, picric acid. ×855.

Fig. 21. Part of the same culture, showing a normal pigmented hair in transverse section. Haematoxylin, chromotrope, picric acid. ×599.

Fig. 22. Part of the same culture, showing on the right a normal heavily pigmented hair and on the left a follicle which is extruding pigment granules. Haematoxylin, chromotrope, picric acid. ×599.



HARDY—THE DEVELOPMENT OF MOUSE HAIR



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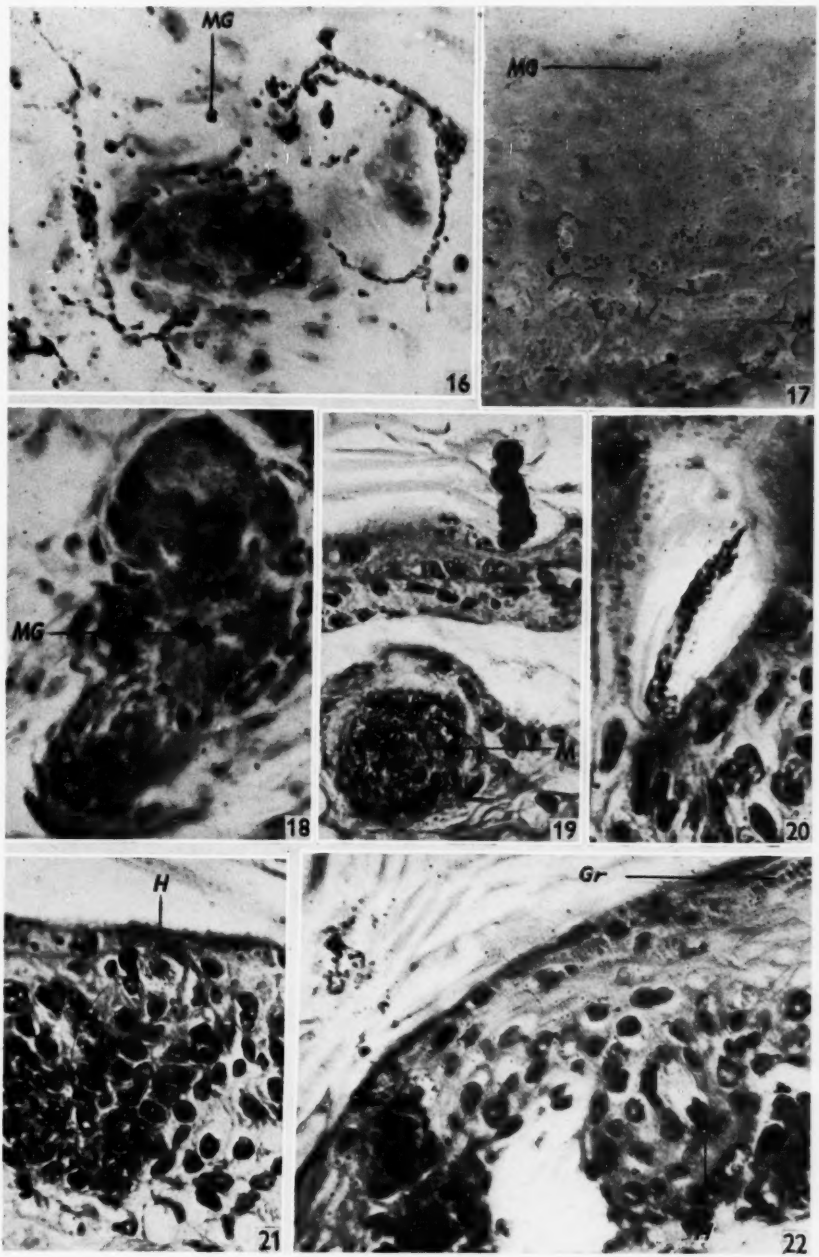

HARDY—THE DEVELOPMENT OF MOUSE HAIR

REVIEWS

Neurological Anatomy in relation to Clinical Medicine. By A. BRODAL. (Pp. xv+496, 80 figs., 3 plates. 1948. 42*s*.) Oxford University Press.

The part which anatomy plays in the field of clinical neurology is well recognized but, in view of the tendency to extreme specialization and the mass of literature which daily accumulates, there is obvious need to review the whole field of neuro-anatomy and to attempt a correlation with neuro-physiology and clinical neurology. This difficult task is ably undertaken by Dr Brodal, who wisely avoids the tendency to produce rigid schemata which so often prove a stumbling block to further progress. Thus he demonstrates not only the extent to which anatomy assists in the understanding of clinical manifestations, but also the points on which academic findings are inadequate, so providing a stimulus to further effort in the anatomical field.

It is implicit in a work of this nature that agreement on all points is not to be expected and that it is impossible for one author to have first-hand knowledge of the whole range of this rapidly expanding subject. Dr Brodal minimizes these difficulties by quoting his sources of information throughout the text and provides an excellent selection of references. These, together with a valuable introductory chapter on the significance of the various neuro-histological techniques, should assist those not directly conversant with the limitations of such procedures to develop a critical approach to the subject.

The book is divided into a series of chapters, each dealing with a separate functional system in terms of its anatomy, physiology and the manifestations of experimental and pathological lesions.

The order in which Dr Brodal presents the various systems is difficult to understand. For example, the introductory chapter is followed by a consideration of the spinal motor neurons. The reader is then plunged into a section dealing with the pyramidal tract and motor area of the cerebral cortex, before reaching the portions concerned with the 'extra-pyramidal' systems and the sensory apparatus. Although it is clear from the text that the author fully appreciates the importance of the 'extra-pyramidal' system in voluntary movements, the above order tends to obscure the close interrelation of pyramidal, extra-pyramidal motor mechanisms and the sensory system in the control of muscular activity. It is interesting that Dr Brodal, in an excellent chapter on the cerebellum, considers first the afferent and then the efferent pathways of this organ with a great increase in clarity compared with the method followed in the book as a whole.

It is perhaps unfortunate that the vascular system with its importance in the origin and development of many pathological processes receives only passing mention. A few minor inaccuracies which have here and there crept into the text might well be altered in later editions. For example, it is stated in relation to the organ of Corti that the apical coil mediates sounds 'of the highest pitch'.

Despite these criticisms and the fact that the clinical descriptions at times outweigh the anatomy, this book should prove of very considerable value to those already acquainted with the gross anatomy of the nervous system. It will appeal to advanced undergraduate and postgraduate students as well as to the clinical neurologist who should find it useful not only for its intrinsic value but also as a source of references for further study.

The book as a whole is pleasantly produced. The printing and reproduction of diagrams and plates is excellent and, despite the fact that much of it is the author's translation of the original publication in the Norwegian language, it is readable and easily comprehended.

G. J. ROMANES

Cunningham's Manual of Practical Anatomy. Revised and edited by JAMES COUPER BRASH, M.C., M.A., M.D., F.R.C.S. Ed., F.R.S.E. 11th ed. vol. 1. (General Introduction, Upper Limb, Lower Limb. Pp. xix+387; 191 figs.); vol. 2 (Thorax and Abdomen. Pp. x+488; 238 figs.); vol. 3 (Head and Neck: Brain. Pp. x+513; 230 figs.). Cr. 8vo. Each vol. 21*s.* net. Oxford University Press: Geoffrey Cumberlege.

Just 70 years after the publication of Prof. Cunningham's *Dissector's Guide* there now appears the 11th edition of his *Manual of Practical Anatomy.* The new edition appears under a single editorship, and in his Preface Prof. Brash pays a graceful tribute to his collaborator in the two previous editions, Dr E. B. Jamieson. Prof. Brash is to be congratulated on achieving the rare distinction of bringing out a new edition with fewer instead of more pages than the previous one.

The main change between this and the 10th edition of 1940 is in the reduction of the number of pages and in the quality of the paper, which have combined to form a series of much smaller volumes. The new edition differs from the reprints of the 10th chiefly in the order of dissection of the brain, which now proceeds from the brain-stem upwards. The new, almost 'art', paper employed gives a bright, white surface to the page, but one fears that it may show, even more than usual, the marks of the wet and greasy fingers of the dissectors, but this is a misfortune that must befall any book brought into the dissecting room. It does seem that this paper is apt not to take the type well, and this is found in several of the figures, particularly the radiographs in vol. 1. On some pages a curious heavier type suddenly appears among ordinary type.

A number of new radiographs of injected vessels have been introduced into vol. 1. Though these show more vessels than the old Plates which they replace, the vessels are almost too numerous and the radiographs are rather dark, so that it is not easy to identify some of the structures.

They are bright, pleasant books to use, neat and handy to slip into the pocket, and the series will surely maintain the high reputation of Cunningham's as a dissecting manual.

C. M. WEST

Evolution of the Forebrain. By G. W. H. SCHEPERS. (Pp. 207 and bibliography. 1948. 50*s.*) Cape Town, South Africa: Maskew Miller, Ltd.

This monograph, which claims to deal with the fundamental anatomy of the telencephalon, is based on the author's study of the structure of the brain of a chelonian reptile, *Testudo geometrica.* About half of it is taken up with a description of the arrangement of nerve cells and fibres and of the blood vascular system in the forebrain of this animal; the rest consists of a very speculative account of the structural and functional principles involved and of the evolution of the forebrain from invertebrate ancestors to man. Considerable use is made of literature earlier than 1935, but there are few references to work later than this date.

The descriptive part of the work is of some value. It is based on the study of serial sections stained by the usual neurological techniques including silver impregnations and Golgi methods. It does not, however, go further than several similar descriptions which have been published for other reptilian brains and there is little evidence for any critical appreciation of the limitations of the methods used. So far as one can tell, since there are few if any references to particular preparations or techniques, the description is based principally on the appearances in a transverse series prepared by a Cajal silver impregnation method, a series which is illustrated by a number of drawings. The other illustrations, which are numerous, are with few exceptions diagrams illustrating the author's morphological and functional interpretations. It is not clear what use, if any, has been made of the Golgi material, and for these reasons among others it is difficult to assess the value of his nterpretations. This is particularly unfortunate, since, from his observations in *Testudo geometrica* the author is frequently led to a reinterpretation of the findings of others in

different reptilian brains with which, apparently, he has no first-hand acquaintance. One may also doubt whether some of the morphological features described, such as shallow grooves on the surface of the brain or small branches of blood-vessels, are either definite or constant enough to carry the weight of theoretical interpretation which is placed upon them.

The second part of the work which attempts to deal with the fundamental principles of forebrain morphology ('morphological fundamentalism', to use the author's term) is too obscure and has too little foundation on observation to merit serious consideration. The argument becomes almost wholly abstract and speculative, and divorced from the materials with which neuro-anatomists are accustomed to work. Detailed criticism is unnecessary.

The author introduces a new or modified terminology for the reptilian brain which seems to have few advantages. There is no index; complicated abbreviations and symbols are used not only for labelling the figures, but also in referring to particular structures in the text, and no alphabetical key to these abbreviations is provided. The book is therefore difficult to read and loses most of any value it might have as a descriptive account of a chelonian brain.

F. GOLDBY

Lehrbuch der Embryologie. By W. BRANDT. (Pp. xii+648; 472 illustrations. 1949. Swiss fr. 56.) Basel: S. Karger.

'Die Entwicklungsgeschichte ist der wahre Lichtträger für Untersuchung über organische Körper.' It is with this quotation from von Baer that Dr Brandt ends the text of this admirable new survey of human development. A reading of the text makes it obvious that the author does not pay mere lip service to the von Baer dictum. He uses the embryological torch not only to illumine adult relationships and developmental abnormalities but also to throw light on the wider biological significance of the facts recorded. Further, and as was to be expected in a book by one who has made important contributions to our knowledge of development, much of the text is first hand and the whole of it is permeated by a real awareness of the biological status of the problems involved.

Dr Brandt divides the subject matter of his volume into two parts, one on general and the other on special embryology. The general part occupies the first 171 pages of the book and deals with such topics as the sex cells, fertilization, cleavage, the germ layers, the organizer mechanism and determination. It also includes an account of the early development of the human embryo, of embryonic and foetal growth and of twinning and descriptions of implantation, of placental development and of general reproductive physiology. This section appears, to the reviewer, to be both succinct and well informed. Indeed, apart from detail, it can only be suggested that the description of menstruation should come before that of implantation and that the account of foetal measurements is a little too detailed.

The second part of the book extends over 456 pages and is concerned with organogenesis and with certain related problems of developmental physiology. It includes an excellent summary of the author's micro-manipulative technique for the experimental study of the development of organs. This section of the book does not appear to be quite as satisfactory as the first. This is doubtless due to the extraordinary difficulty, with the present embarrassing richness of our knowledge of descriptive embryology, of presenting organogeny in a well-balanced manner. Personal knowledge of and, in a sense, special affection for certain organs or regions is bound to result in an over-emphasis of some aspects at the expense of others. Nevertheless, the reader of this section will find much that is new, at least to those who have not followed the German literature of the last twenty years, and he will be stimulated in particular by the descriptions of the determination problem as it affects, for example, the development of the gut and its derivatives and of the joints.

So far as the reviewer can judge the text is well written. At any rate the German is not difficult to follow. The book is well printed and produced and many of the figures are excellent. A great defect, however, is the absence of a bibliography. This absence is the more surprising, and tantalizing, in that there are many author references, with dates, in the text. There is, however, a good subject index.

J. D. BOYD

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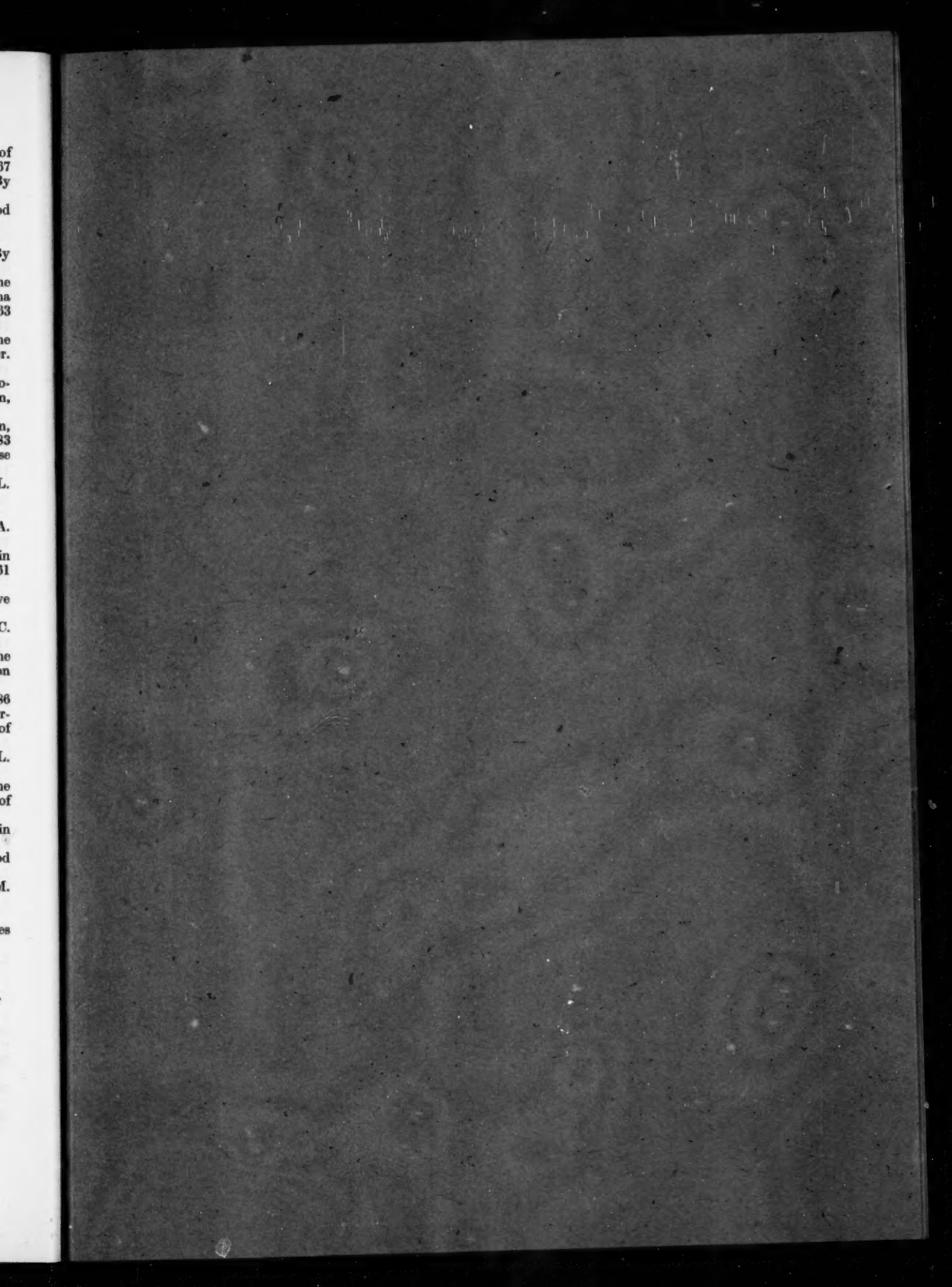

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